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Studies of immobilized glucose oxidase on galvanostatically synthesized poly(*N*-methylpyrrole) film with PVS-$NaNO_3$ composite dopant

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Incorporation of the glucose in presence of phosphate and acetate buffer were studied to investigate the poly(*N*-methylpyrrole)/polyvinyl sulfonic acid/sodium nitrate/glucose oxidase (P(NMP)/PVS/$NaNO_3$/GODx) electrode. The P(NMP)/PVS/$NaNO_3$ films were electrochemically synthesized and characterized by using electrochemical technique, FTIR and SEM. Glucose oxidase was immobilized by cross-linking via glutaraldehyde on the galvanostatically synthesized poly(*N*-methylpyrrole) /polyvinyl sulfonic acid/sodium nitrate (P(NMP)/PVS/$NaNO_3$) film. The higher sensitivity of P(NMP)/PVS/$NaNO_3$/GODx electrode was recorded in phosphate buffer than that of acetate buffer and that was found to be 4 µA/mM and 2.2 µA/mM, respectively. The kinetics parameters of P(NMP)/PVS/$NaNO_3$/GODx electrode were determined for the phosphate buffer and acetate buffer. In phosphate buffer the observed values of K_m and I_{max} were 14.4 mM and 111.2 µA, respectively and in acetate buffer 16.9 mM and 68 µA, respectively. The stability of synthesized P(NMP)/PVS/$NaNO_3$/GODx electrode in phosphate buffer was found to be more than that of acetate buffer.

Keywords: *N*-methylpyrrole, composite dopant, galvanostatic, glucose-oxidase electrode

1. INTRODUCTION

Electrochemically synthesized polymers are being extensively used as enzyme immobilization matrix for the development of biosensors [1-6]. Conducting polymers are capable of incorporating different functionalities in their matrix during or after polymerization. It is well known that the properties of synthesized polymer films are affected by electropolymerization condition. Many researchers have

extensively studied the synthesis of polymer films, which can be used as a polymer matrix for immobilization of biocomponents [7-10].

Various conducting polymers have been considered for immobilization of enzymes [11-15]. However, polypyrrole and its family have gained most interest for the immobilization of enzyme, because of its low oxidation potential, environmental stability, sensitivity and good quality matrix. These characteristics enable the growth of film from aqueous solutions that are compatible with most biological systems [16-17]. Moreover, its easy polymerization, high electrical conductivity, chemical stability and ability to form freestanding film are added advantages for its application to biosensors.

The estimation of glucose in blood is an important parameter for the diagnosis and prevention of diabetic's disease. Several biosensors with the enzymatic method have been reported [18-21]. However, still it is essential to continue the research in this field with new material and approach, so that the sensitivity and stability of the sensor can be improved. In the present investigation, we have synthesized the P(NMP) matrix with composite dopant PVS and $NaNO_3$ for the development of biosensors. The PVS plays vital role to avoid leaching of immobilized enzyme, which leads to the stability of biosensors and $NaNO_3$ ions mobility is very high, which leads to the higher sensitivity of biosensor.

The GODx was immobilized on the P(NMP)/PVS/$NaNO_3$ matrix by cross-linking via glutaraldehyde. Long lifetime stability of the enzyme over the matrix is the vital factor in the development of biosensor. The major cause of poor stability is the disorption (leaching out) of enzyme from immobilization materials. Therefore, to overcome this problem cross-linking method via glutaraldehyde has been chosen for the immobilization of enzyme in the present investigation. The P(NMP)/PVS/$NaNO_3$/GODx electrode was tested in phosphate and acetate buffer and the sensitivity and stability of the biosensor have been compared.

2. EXPERIMENTAL

2.1 Synthesis of P(NMP)/PVS/$NaNO_3$ film

The P(NMP)/PVS/$NaNO_3$ film was synthesized from an aqueous solution of 0.05 M *N*-methylpyrrole (NMP) (Aldrich), 0.025 M sodium salt of polyvinyl sulfonic acid (25 % by weight) (Aldrich) and 0.05 M sodium nitrate ($NaNO_3$) (Rankem) using electrochemical deposition method. It was carried out by galvanostatic technique at 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/cm^2 and the pH 1.5 were kept constant during synthesis of P(NMP)/PVS/$NaNO_3$ films. After synthesis the polymer coated electrodes were rinsed thoroughly in deionized water dried in cold air and then use for subsequent characterization.

The characterization of synthesized P(NMP)/PVS/$NaNO_3$ film was carried out by FTIR spectra (FTIR-8400 Shimadzu) and scanning electron micrographs (JEOL, JSM-6360A, Analytical SEM).

2.2 Immobilization of GODx on P(NMP)/PVS/$NaNO_3$ films

The stock solution of glucose oxidase (GODx) (EC 1.1.3.4, Type VII) (200 U/ml) (Aldrich) prepared in 0.1 M phosphate or acetate buffer (pH 7.4) was adsorbed onto the surface of P(NMP)/PVS/$NaNO_3$ films. The enzyme GODx was immobilized by cross-linking via (0.1 %) glutaraldehyde (Loba Chemie) on P(NMP)/PVS/$NaNO_3$ films left for 30 min and washed 2-3 times with phosphate or acetate buffer to remove loosely bound enzyme if any.

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 will be more accessible for the enzymatic reaction. The lifetime of the biosensor was studied when it was kept at (4 °C) in phosphate and acetate buffer. An adequate concentration of GODx and glutaraldehyde in cross-linking mixture were chosen so that it ensure higher enzyme loading and provide excellent amperometric response with an efficient retention of the enzyme.

2.3 Determination of Glucose

The stock solution of D-glucose (50 mg/ml) was prepared in phosphate buffer (0.1 M, pH 7.4) and acetate buffer (0.1 M, pH 7.4) and left for 24 hours before testing. To generate calibration plots of response current versus time for the glucose concentrations, 0.02 ml of D-glucose solution were injected for every 50 seconds into the electrochemical cell containing 55 ml of (0.1 M phosphate buffer) solution, resulting in 1 mM glucose increments. The rate of increment of glucose concentration was 1 mM from 1 mM to 5 mM, it was 5 mM from 5 mM to 10 mM and 10 mM from 10 mM to 50 mM. The average steady state current was recorded at each step of glucose concentration. The electrode was removed from the solution and washed with fresh respective buffers and kept at 4 °C in order to use it for another assay.

3. RESULTS AND DISCUSSION

3.1 Galvanostatic studies of P(NMP)/PVS/$NaNO_3$ films

The potential-time curve of synthesized P(NMP)/PVS/$NaNO_3$ film with 1 mA/cm^2 current density, pH 1.5 , 0.05 M *N*-methylpyrrole, 0.025 M PVS and 0.05 M $NaNO_3$ at 27 °C is shown in **Fig. 1.** The behavior of the galvanostatic synthesis overshoot during the first few seconds probably indicates the difficult formation of dimmers and oligomers. After this, potential becomes almost constant suggesting

that building up of the film proceeds according to the same reaction along the full thickness of the polymer. Since the anion of PVS has large size, it will not leave the polymer matrix easily, the $NaNO_3$ ions has small size, which may leads to the good stability and conductivity of the synthesized film.

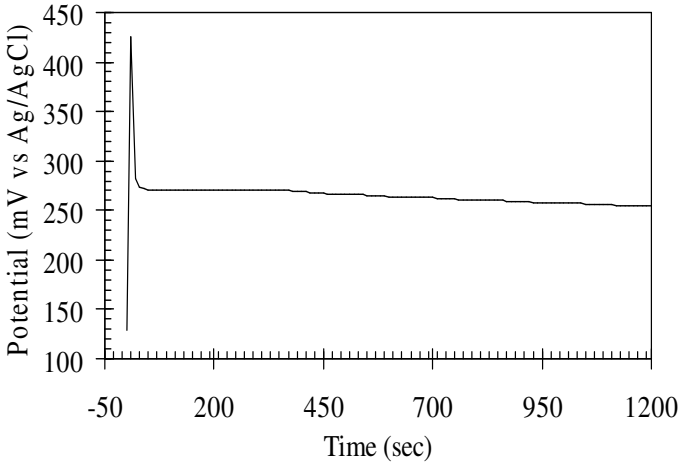

Figure 1. Potential-time curve of synthesized P(NMP)/PVS/$NaNO_3$ film with 1 mA/cm^2 current density and pH 1.5

3.2 FTIR study of P(NMP)/PVS/$NaNO_3$ films

The FTIR spectra recorded for the synthesized P(NMP)/PVS/$NaNO_3$ film at 1 mA/cm^2 current density and 1.5 pH is shown in **Fig. 2**. The absorption band observed at 2923 cm^{-1} corresponds to the C-H stretching of CH_3 of P(NMP). The bands at1444.6 and 1265.2 cm^{-1} were due to the ring stretching of P(NMP). The bands at 1033.8 cm^{-1} was for C-H in plane deformation and the C-H out of plane deformation was observed at 887.2 cm^{-1}. The bands observed at 1375 and 1450 cm^{-1} were due to the C-H (methyl). The N-H stretching was observed in the range 3225-3192 and 1590-1655 cm^{-1}. The band at 1045 cm^{-1} corresponds to the stretching of SO_3^- group. The band at 1695.2 cm^{-1} was observed for the C=O stretch of acetate group. The FTIR spectra showed a good resemblance with earlier reported work [22, 23].

3.3 SEM study of P(NMP)/PVS/$NaNO_3$ film

The surface morphology of the P(NMP) film synthesized with PVS-$NaNO_3$ composite dopant at 1 mA/cm^2 current density and pH 1.5 was carried out by scanning electron microscope (SEM) (**Fig. 3**). It shows the porous and cauliflower like matrix, which is suitable for the incorporation of the enzyme. The porous matrix certainly enhances the sensitivity of the glucose biosensor, because it can entrap the biocomponent/enzyme easily and can hold it for longer duration.

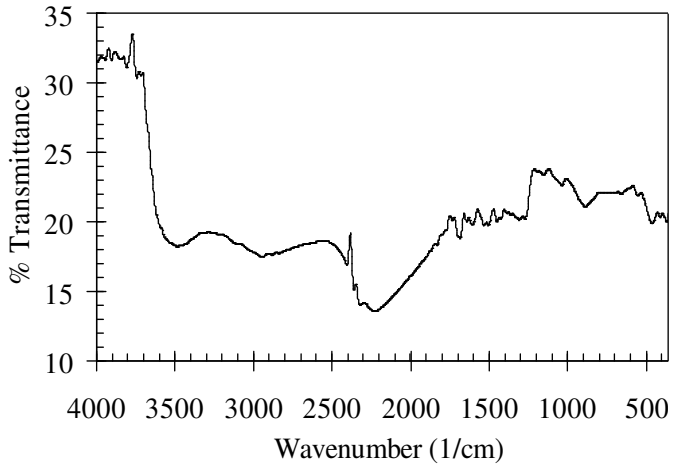

Figure 2. FTIR spectra of synthesized P(NMP)/PVS/$NaNO_3$ with 1 mA/cm^2 current density and pH 1.5

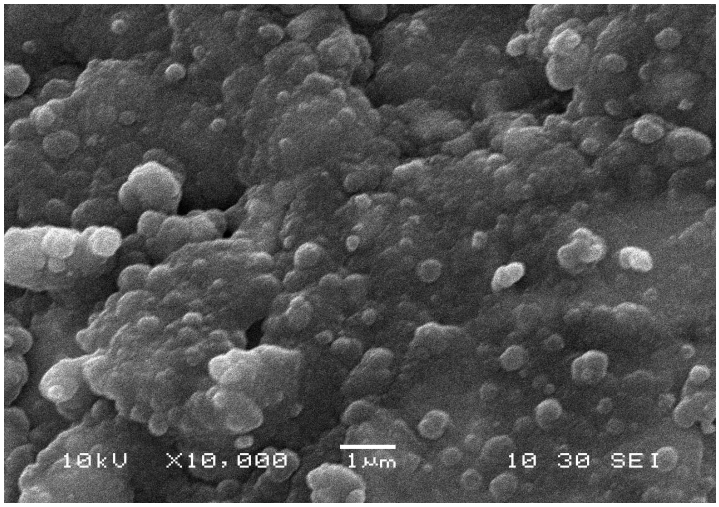

Figure 3. SEM of synthesized P(NMP)/PVS/$NaNO_3$ with 1 mA/cm^2 current density and pH 1.5

3.4 Response current of P(NMP)/PVS/$NaNO_3$/GODx electrode

The activity of the immobilized GODx can be easily evaluated by the electrochemical analysis; the amperometric current is the identity of the activity of the immobilized GODx which is proportional to the concentration of the H_2O_2, a product produced by the GODx on the anode. It can be stated as,

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

Thus, with the sensing current we can determine the activity of immobilized GODx. The formation of hydrogen peroxide is detected by the amperometric 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, 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 [24]. The electron transfer from the redox cofactor to the sensing electrode is also facilitated by the presence of a polymeric conducting material.

The response current of P(NMP)/PVS/$NaNO_3$/GODx electrode for different concentrations of glucose (1 mM – 50 mM) in phosphate buffer (pH 7.4) and acetate buffer (pH 7.4) at 0.7 V potential with time is shown in **Fig. 4** and **Fig. 5** respectively. It was observed that the response current increases

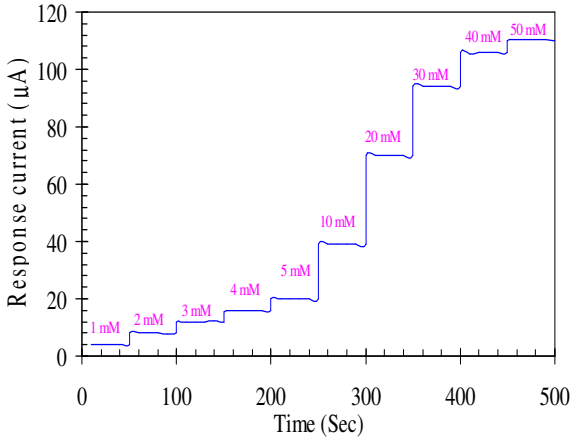

Figure 4. Response current-time curve for the P(NMP)/PVS/$NaNO_3$/GODx electrode in phosphate buffer (pH 7.4) for different glucose concentration

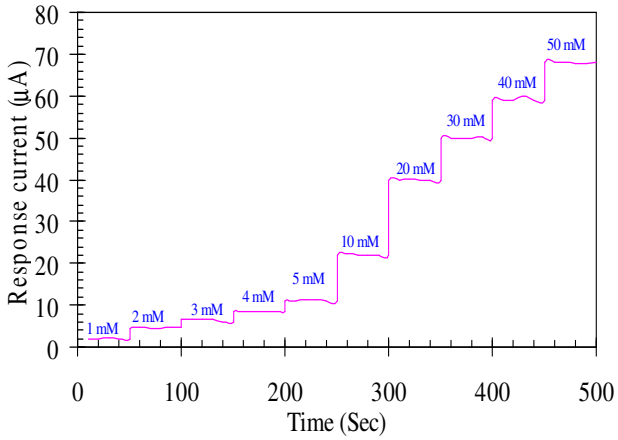

Figure 5. Response current-time curve for the P(NMP)/PVS/$NaNO_3$/GODx electrode in acetate buffer (pH 7.4) for different glucose concentration

with increase in the concentration of glucose for phosphate as well as acetate buffer. The average steady state current was recorded at each step in glucose concentration. The relationship between the response current and glucose concentration for potential 0.7 V in phosphate buffer as well as acetate buffer is shown in **Fig. 6** and **Fig. 7**. It was observed that, current increases with increasing glucose concentration in the range (1 mM – 50 mM). It exhibits a good linearity for sensing glucose in the range 1 mM to 10 mM (inset in Fig 6 and 7).

3.5 Determination of Michaelis-Menten constant (K_m)

The Michaelis-Menten constant (K_m) was calculated for the immobilized enzyme. The plot of 1/current vs 1/glucose concentration at potential 0.7 V in phosphate buffer and acetate buffer is shown in Fig. 8. In phosphate buffer (pH 7.4) the maximum current (I_{max}) is 111.2 µA with K_m 14.4 mM and for the acetate buffer (pH 7.4) the maximum current (I_{max}) is 68 µA with K_m 16.9 mM for potential 0.7 V. Moreover, we found higher sensitivity for phosphate buffer than that of acetate buffer **(Table 1).**

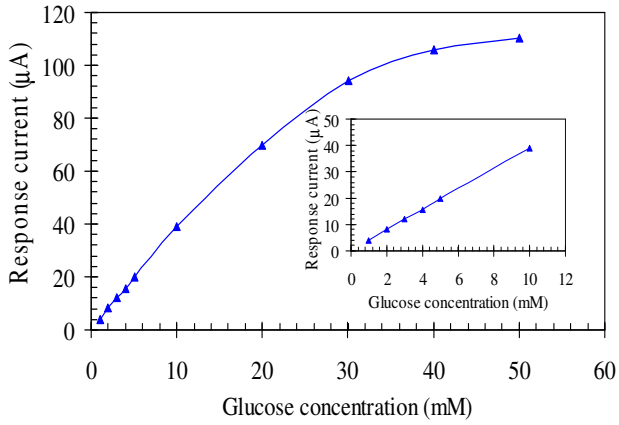

Figure 6. The relationship between response current and glucose concentration for the P(NMP)/PVS/$NaNO_3$/GODx electrode in phosphate buffer (pH 7.4) at potential 0.7 V

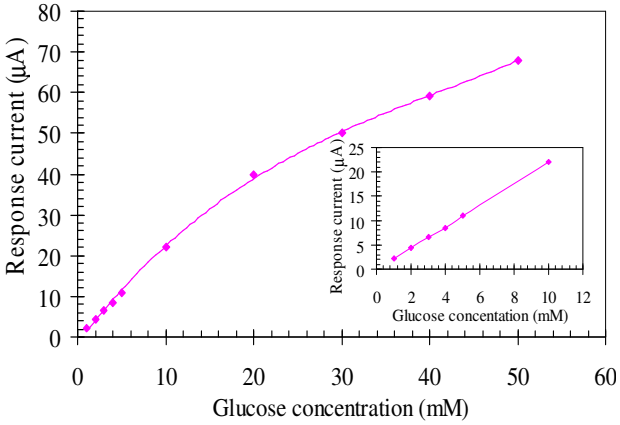

Figure 7. The relationship between response current and glucose concentration for the P(NMP)/PVS/$NaNO_3$/GODx electrode in acetate buffer (pH 7.4) at potential 0.7 V

The value of K_m depends on the immobilization of enzyme lesser value of K_m gives higher affinity between substrate and the enzyme, which ultimately gives faster response [25]. Hence, the phosphate buffer should be preferred for the immobilization of GODx.

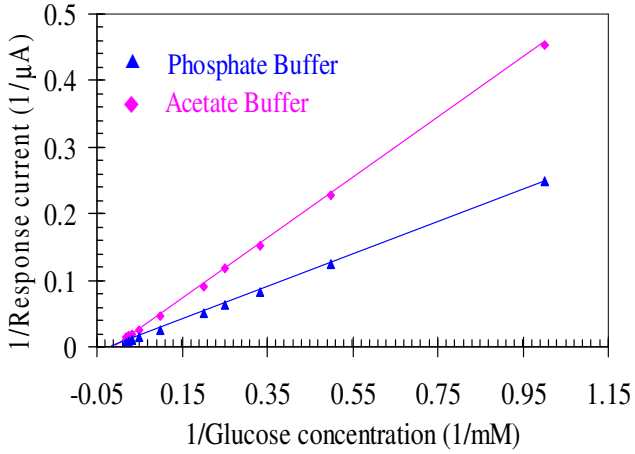

Figure 8. Determination of Michaelis-Menten constant (K_m) for P(NMP)/PVS/$NaNO_3$/GODx electrode in phosphate and acetate buffer (pH 7.4)

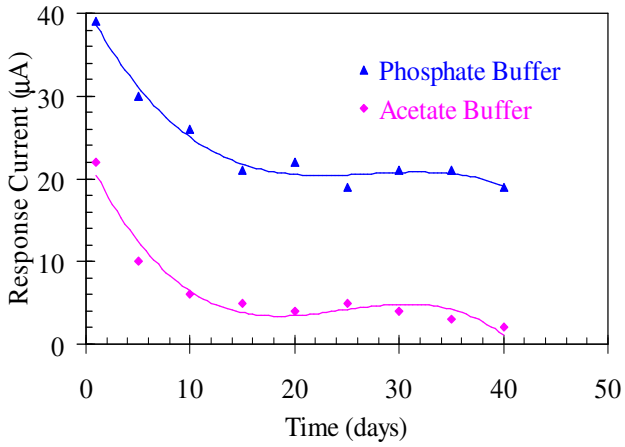

Figure 9. The stability of synthesized P(NMP)/PVS/$NaNO_3$/GODx electrode for 10 mM of glucose concentration.

Table 1
Comparison of the analytical performance of P(NMP)/PVS/$NaNO_3$ /GODx electrode for phosphate and acetate buffer at pH 7.4.

Sr. No	Parameters	Buffers	
		Phosphate	Acetate
1	I_{max} (μA)	111.2	68
2	K_m (mM)	14.4	16.9
3	Linearity (mM)	1-10	1-10
4	Sensitivity (μA/mM)	4	2.2
5	Lifetime (days)	35	32

3.6 Stability of the P(NMP)/PVS/$NaNO_3$/GODx electrode

The stability of the synthesized P(NMP)/PVS/$NaNO_3$/GODx electrode has been tested for both buffers (**Fig. 9).** It was observed that in the beginning the response current decreases which became more stable later. Again the current response of synthesized P(NMP)/PVS/$NaNO_3$/GODx electrode in acetate buffer decreases much more rapidly than that of phosphate buffer. The long-term stability was carried out for 40 days for both buffers. It was found that the P(NMP)/PVS/$NaNO_3$/GODx electrode exhibited excellent stability for 35 days in phosphate buffer and 32 days in acetate buffer.

4. CONCLUSIONS

The P(NMP)/PVS/$NaNO_3$ films have been successfully synthesized on ITO coated glass plate that was confirmed by the FTIR study. The SEM of the P(NMP)/PVS/$NaNO_3$ film showed porous and cauliflower like structure which is suitable for entrapment of the GODx. The immobilization of GODx on the galvanostatically synthesized P(NMP) film with composite dopants PVS and $NaNO_3$ by cross-linking via glutaraldehyde has been successfully carried out. The sensitivity of P(NMP)/PVS/$NaNO_3$/GODx electrode was found to be higher for phosphate buffer, it also followed the kinetics parameters. The P(NMP)/PVS/$NaNO_3$/GODx electrode showed excellent response in phosphate buffer (7.4 pH) and also excellent stability for 35 days.

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