Journal of Applied Sciences

ISSN 1812-5654

science alert

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Journal of Applied Sciences 9 (10): 1907-1914, 2009
ISSN 1812-5654
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Development of a Polypyrrole-Based Amperometric Phosphate Biosensor

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Abstract: A biosensor is developed by co-entrapment of Purin Nucleoside Phosphorylase (PNP) and xanthine oxidase (XOD) and potassium ferrocyanide ($K_4Fe(CN)_6$ into a polypyrrole (PPy) film via., galvanostatic polymerisation of pyrrole. The optimum conditions for formation of the Ppy-PNP-XOD-$Fe(CN)_6^{4-}$ film are 0.3 M pyrrole. 6.2 U mL^{-1} XOD, 49 U mL^{-1} PNP, 40 mM $K_4Fe(CN)_6$, polymerisation period of 200 sec and an applied current density of 0.5 mA cm^{-2}. The optimum potential for the amperometric biosensing of phosphate was 200 mV vs. Ag/AgCl (3 M KCl) in 0.05 M barbitone buffer. The achievable linear concentration range was between 0.1 and 1 mM, while the minimum detectable amount was 10 μM.

Key words: Biosensor, bienzyme, pyrrole, xanthine oxidase, purine nucleotide phosphorylase amperometric measurement

INTRODUCTION

Phosphate determination is of importance in environmental analysis because of its widespread presence in detergent and fertiliser, which can pollute natural water (Keup, 1968). The eutrophication of water by phosphate and nitrogen can lead to over-growth of plant and toxic algae, thereby making it unsuitable for drinking or industrial use (Gather and Nagatiah *et al*., 1998). The adverse effect of excess intake of phosphate in food additives upon human health has also become of interest in food analysis (Nordin, 1997). In clinical diagnosis, the determination of phosphate in body fluid provides useful information about some diseases, the energetic state of cells and bone function.

The analytical methods commonly employed for determination of phosphate are spectrophotometric (Mckelvie, 2000) and chromatographic methods (Bello and González, 1996; Colina *et al*., 1996), however these require sample pre-treatment, are time consuming and produce toxic wastes (for example, tartaric acid and heavy metals such as antimony). The use of ion selective electrodes has also been reported for phosphate determination, based on Sn complexes (Chaniotakis *et al*., 1993), hydroxyapatite (Petrucelli and Kawachi *et al*., 1996) and Copper wire or cobalt metallic wires (Chen *et al*., 1997; De Marco and Phan, 2003). However, these electrodes suffer from low selectivity and poor stability of the ion selective membranes. Recently A biosensor for inorganic phosphate using rhodamine labeled phosphate binding protein was developed by Okoh *et al*. (2006). Another novel polyvinyl chloride (PVC) matrix membrane sensor responsive to inorganic phosphate ion was described by Lin *et al*. (2006) while and Akylmaz and Yorgancia (2007) developed biosensor based on the pyruvate oxidase modified conducting polymer for phosphate ions determinations. Pyruvate oxidase was also used by other researchers to determine phosphate ion (Gavalas and Chaniotakis, 2001; Ikebukuroa *et al*., 1996; Rahman *et al*., 2006; Roger *et al*., 2005). Flow injection Molybdate method has recently been developed by Motomisu and Li (2005), Yaqoob *et al*. (2004), Mecozzi (1995) and Galhardo and Macini (2000) for nanomolar of phosphate determination. While conductometry method was also recently used to determine phosphate (Zhanga, 2008).

A rapid and simple alternative is the use of an enzyme sensor that is able to measure phosphate directly in the sample. Various phosphate biosensors have been developed based on enzymatic sequences in which a first enzyme (usually a phosphorylase) uses phosphate as a co-substrate giving a product that is the substrate for a second enzyme, usually an oxidase. Among these are phosphate biosensors that use, as biorecognition elements, substances such as Purin Nucleoside Phosphorylase (PNP) and xanthine oxidase (XOD) as in (D'Urso and Coulet, 1990, 1993; Male and Luong, 1991; Kulys *et al*., 1992; Wollemberger and Schuber, 1992), alkaline or acid (Guilbault and Nanjo, 1975; Guilbault, 1984), phosphatase and glucose oxidase (Su and Macini, 1995), pyruvate oxidase (Ikebukuroa *et al*., 1996), sucrose phosphorylase (Conrath *et al*., 1995)

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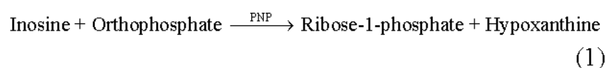
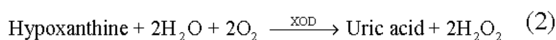
phosphoglucomutase and glucose 6-phosphate dehydrogenase (Conrath *et al*., 1995). In these studies optical (chemiluminescent) (Ikebukuro *et al*., 1996) and electrochemical (amperometric) (D'Urso and Coulet, 1990; Male and Luong, 1991; Kulys *et al*., 1992; Wollemberger *et al*., 1992; D'Urso and Coulet, 1993) transducers were used. The advantages and disadvantages of the biosensors for the determination of phosphate have been reviewed by Engblom (1998). Recently, a biosensor for phosphate was fabricated using a combination of maltose phosphorylase (MP), mutarotase (MR) and glucose oxidase (GOx) entrapped in an inorganic laponite clay (Mousty *et al*., 2001; Su and Mascini, 1995) The response of the biosensor for phosphate detection was measured by applying a potential of 0.6 V vs. Ag/AgCl to the electrode in order to oxidise the enzymatically generated hydrogen peroxide (Mousty *et al*., 2001).

In order to lower the detection limit, amplification by enzymatic substrates recycling was employed by Wollenberger and Schuber (1992), involving co-immobilisation of alkaline phosphatase (aP) and glucose oxidase. In the presence of phosphate ion inosine is phosphorylated by PNP to ribose-1-phosphate from which phosphate is liberated by aP catalysis and is thus again available for inosine phosphorylation. Phosphate is thus recycled between aP and PNP while a higher amount of hypoxanthine is produced and recognised by sequential oxidation by XOD (Wollemberger and Schuber, 1992).

In this study, an amperometric biosensor was developed based on the enzymatic reaction shown in Eq. 1 and 2:



$$\text{Inosine} + \text{Orthophosphate} \xrightarrow{\text{PNP}} \text{Ribose-1-phosphate} + \text{Hypoxanthine} \quad (1)$$



$$\text{Hypoxanthine} + 2H_2O + 2O_2 \xrightarrow{\text{XOD}} \text{Uric acid} + 2H_2O_2 \quad (2)$$

The enzyme, PNP, which reacts only in the presence of inorganic phosphorus, was immobilised together with XOD into polypyrrole films. Male and Luong (1991), D'Urso and Coulet (1990, 1993), Kulys *et al*. (1992) and Guilbault and Nanjo (1975) used the same enzymatic reaction, as in Eq. 1 and 2 to determine phosphate. The successful immobilisation of XOD into polypyrrole film in our previous paper (Lawal and Adeloju, 2008) suggests that co-immobilisation with PNP is feasible. To this end, the optimisation of the PPy-PNP-XOD biosensor in this study involved the investigation of the effect of film thickness, pyrrole concentration, enzyme (PNP:XOD) ratio, applied potential, pH, inosine and buffer concentrations.

MATERIALS AND METHODS

Reagents and standard solutions: XOD (EC.1.2.3.2.2 Grade1) from buttermilk, purine nucleoside phosphorylase (PNP) (EC.2.4.2.1), potassium ferrocyanide, pyrrole and hypoxanthine were obtained from Sigma. Other chemicals used were analytical reagent grade and all compounds used in this work were prepared without further purification. Barbitone buffer stock solution (0.5 M, pH 7.8) was prepared by neutralising 0.5 M barbituric acid with 0.1 M sodium hydroxide. This was stored in a refrigerator and diluted when needed.

Apparatus: Electrochemical measurements were made with a potentiostat/galvanostat designed and constructed in our laboratories. This instrument was used in galvanostatic mode for the electropolymerisation. A three-electrode system, including platinum working electrode, a platinum wire counter electrode and an Ag/AgCl reference electrode, was employed for amperometric detection of phosphate. The potentiostat was connected to a computer controller system. The solution was stirred when necessary with a Sybron Thermolyne (model S-17410) stirrer.

Preparation of PPy-PNP-XOD electrode

Electropolymerisation of PPy-PNP-XOD film: A three-electrode voltammetric cell was used to perform electropolymerisation of the PPy film. Platinum wire and Ag/AgCl (3 M KCl) were used as the auxiliary and reference electrodes, respectively while the working electrode was a platinum electrode (0.17 cm^2). The PPy-PNP-XOD-$Fe(CN)_6^{4-}$ electrode, used as a phosphate biosensor, was made by immobilising XOD (6.2 U mL^{-1}), PNP (48 unit mL^{-1}) and 50 mM potassium ferrocyanide ($K_4Fe(CN)_6$) into a polypyrrole film. The electropolymerisation was accomplished in the presence of pyrrole monomer (0.1-0.5 M) at a chosen current density (0.125-0.5 mA cm^{-2}) and polymerisation time of 200 sec. After the galvanostatic film formation, the polymer electrode was washed several times under a stream of Milli-Q water to remove any weakly bound XOD or PNP or $K_4Fe(CN)_6$ molecules prior to analysis.

Amperometric measurements: A three-electrode (10 mL) electrochemical cell was used with an Ag/AgCl (3M NaCl) reference electrode and a platinum wire auxiliary electrode. The working electrode was the PPy-PNP-XOD-$Fe(CN)_6^{4-}$ electrode. The supporting electrolyte used for the amperometric measurements was a 0.05 M barbitone buffer solution (pH 7.8) which contained 0.1 M sodium chloride and 10 mM inosine. The solution in the electrochemical cell was stirred with a magnetic stirrer. A

potential of 0.2 V was then applied to the electrode and the current response to the addition of a phosphate standard solution was recorded.

RESULTS AND DISCUSSION

Response to phosphate: The reactions involved in the phosphate biosensor are illustrated in Eq. 1 and 2. PNP catalysed the phosphorylation of inosine to ribose 1-phosphate hypoxanthine. XOD in the presence of molecular oxygen oxidised Hx and produced hydrogen peroxide which was detected by PPy-PNP-XOD-$Fe(CN)_6^{4-}$ electrode. The reduced mediator was simultaneously regenerated on the electrode surface giving an amperometric signal directly proportional to phosphate concentration.

Figure 1 shows that the amperometric response increased with increasing phosphate concentration. This confirms that the enzymes (PNP and XOD) were successfully entrapped within the polypyrrole film. Thus, the response time was controlled by the polypyrrole film thickness and polymer permeability. The attainment of optimal thickness of polypyrrole film is therefore desirable. The influence of other parameters was subsequently investigated to obtain optimum response for phosphate with the PPy-PNP-XOD-$Fe(CN)_6^{4-}$ electrode.

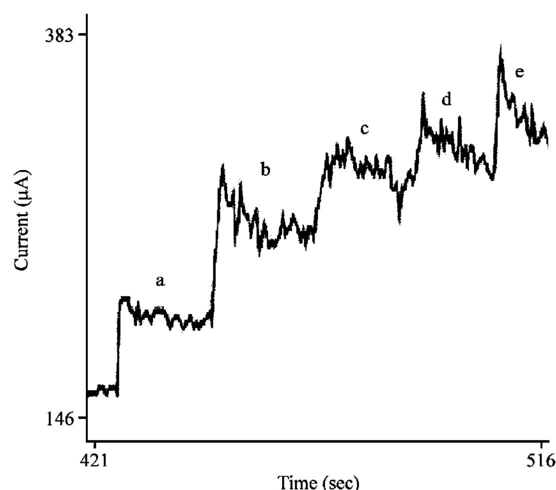


Fig. 1: Typical amperometric response of PPy-PNP-XOD-$Fe(CN)_6^{4-}$ biosensor to phosphate. (a) 10, (b) 20, (c) 30, (d) 40 and (e) 50 mM phosphate. The monomer solution contained 0.4 M pyrrole, current density: 0.5 mA cm^{-2} polymerisation period: 200 sec and E_{app}.200 mV

Effect of PNP and XOD ratio: The amount of XOD in the pyrrole solution was kept constant at 6.2 U mL^{-1} and the amount of PNP was varied to determine the optimal XOD:PNP ratio for the formation of PPy-XOD-PNP-Fe $(CN)_6^{4-}$ film. Figure 2 shows that the optimum response for phosphate with the bienzyme electrode was obtained in the presence of 48 U mL^{-1}, which corresponds to a XOD: PNP ratio of 1:8. This is in agreement with the previous XOD:PNP ratio reported by D'Urso and Coulet (1990, 1993) for a phosphate biosensor fabricated by immobilisation of XOD and PNP on an acetate membrane. Guilbault and Nanjo (1975) reported a ratio of 1:10, while Kulys *et al.* (1992) and Wollenberg and Schuber (1992) reported a ratio of 1:5 for their construction of a phosphate biosensor by immobilisation with GLA and BSA on nylon. On the other hand, Yao *et al.* (2003) and Yao and Wasa (1998) reported an optimum ratio of 1:21, while Konisita *et al.* (1995) found a ratio of 1:3 to be the optimum ratio for immobilisation with BSA/GLA on a reactor. Different choices of membrane and immobilisation methods may account for the observed differences in the enzyme ratios used for the phosphate biosensors based on the use of the bienzyme system.

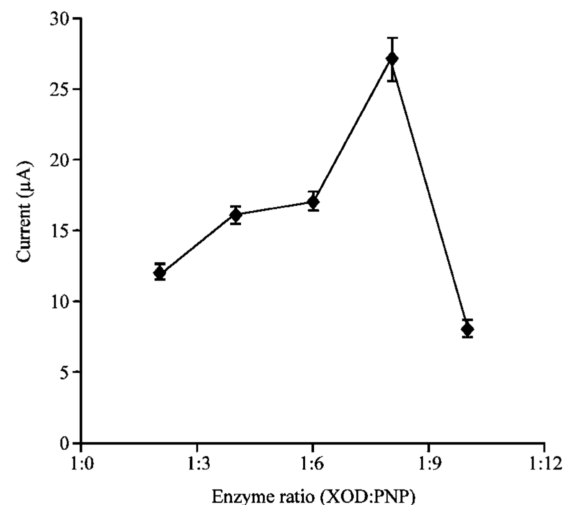


Fig. 2: Effect of immobilised [PNP] on phosphate response with PPy-PNP-XOD-$Fe(CN)_6^{4-}$ electrode. [XOD] was 6.2 U mL^{-1}, $Fe(CN)_6^{4-}$ was 50 mM and [Phosphate] was 10 mM. The monomer solution contained 0.3 M pyrrole, current density: 0.5 mA cm^{-2} polymerisation period: 200 sec and E_{app}.200 mV

Effects of pH and inosine concentration: Figure 3 shows the effect of pH on the response of phosphate on the PPy-PNP-XOD electrode. Xanthine oxidase has an iso-electric point at pH 7.8 (Berger and Grass, 1989) and nucleoside phosphorylase at pH 7.4 (Berger and Grass, 1989), but the optimum pH of the sensor was found to be between 7 and 8 close to the optima of the two enzymes in solution (Berger and Grass, 1989). No response to

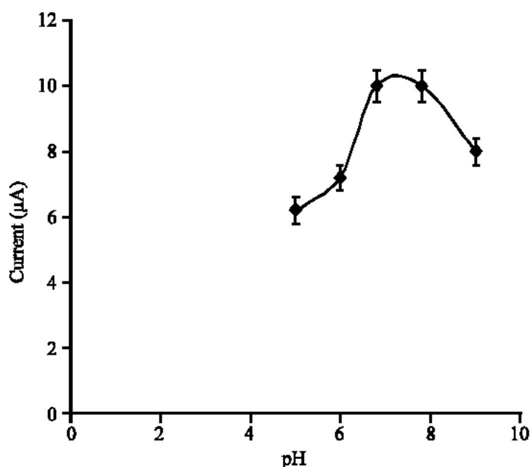


Fig. 3: Effect of pH on phosphate response obtained with the PPy-PNP-XOD- $Fe(CN)_6^{4-}$ electrode. [Phosphate] was 10 mM. The monomer solution contained 0.4 M pyrrole, 6.2 U mL^{-1} of XOD, current density: 0.5 mA cm^{-2} polymerisation period: 200 sec 40 mM $Fe(CN)_6^{4-}$ and E_{app}.200 mV

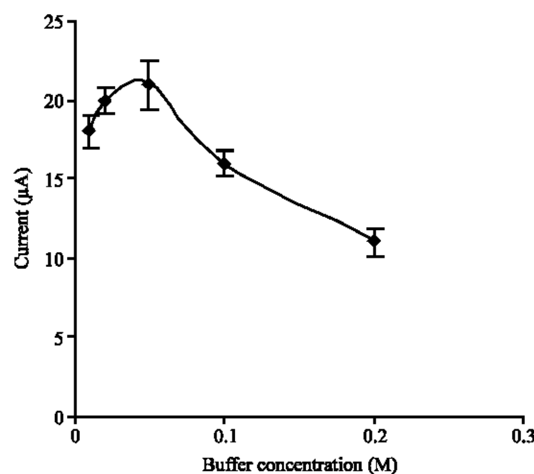


Fig. 4: Effect of buffer concentration on phosphate response obtained with PPy-PNP-XOD-Fe $(CN)_6^{4-}$ electrode. [Phosphate] was 10 mM. The monomer solution contained 0.3 M pyrrole, 6.2 U mL^{-1} of XOD, current density: 0.5 mA cm^{-2} polymerisation period: 200 sec and 50 mM $Fe(CN)_6^{4-}$ and E_{app}.200 mV

phosphate was obtained when either one or both PNP and XOD were not included in the immobilisation or if the sample did not contain inosine, which had to be supplied in excess in a phosphate-free buffer. Barbitone buffer was chosen for the measurement in the presence of excess inosine (D'Urso and Coulet, 1993). Wollenberger and Schuber (1992) also used excess inosine to ensure a co-reactant independent of phosphate response was obtained. Guilbault and Nanjo (1975) and Kulys *et al.* (1992) found that pH 7 gave the highest sensitivity, while D'Urso and Yao (1988) and Yao *et al.* (2003) found pH 7.5 to be optimum.

The chosen optimum inosine concentration for obtaining the most sensitive amperometric response for phosphate was 10 mM. Further increase in inosine concentration did not increase the sensitivity of the phosphate response. It is important to note that no response was obtained when the solution did not contain inosine. D'Urso *et al.* (1990, 1993) used 0.1 mM of inosine, while Kulys *et al.* (1992) used 5 mM of inosine in 0.05 M borate buffer. Wollenberger and Schuber (1992) used 0.09 mM, while the amount of ionsine used by Watanabe *et al.* (1987) was not specified, but inosine was used in excess in 0.05 M Tris-HCl buffer solution. Guilbault *et al.* (1984) used inosine concentration of 5 mM in 0.05 M tris-HCl buffer at pH 7.0. The optimum inosine concentration of 10 mM established in this study, was higher possibly due to the immobilisation of the active enzymes in polypyrrole film, but was still close to the levels used in some studies (Kulys *et al.*, 1992).

Effect of buffer concentration: From Eq. 1 and 2, when PNP catalyses the phosphorylysis of inosine in presence of phosphate to ribose-1-phosphate and hypoxanthine, the hypoxanthine is oxidised; H_2O_2 and uric acid are produced. The H_2O_2 and uric acid are electrochemically active and, hence, they can be easily detected. A signal could be obtained due to change in pH caused by the production of uric acid. Optimisation of the buffer concentration is therefore essential.

Different buffer solutions that have been used for biosensing of phosphate. D'Urso and Coulet (1993) and Watanabe and Endo (1988) used barbitone and borate buffer, while Gaulbault and Nanjo (1975) used 0.01 M Tris HCl. Watanabe *et al.* (1987) and Kulys *et al.* (1992) used borate and citrate buffer solutions, respectively. In this study, we have chosen to use barbitone buffer because it gave stable and sensitive phosphate response. Figure 4 shows that a buffer concentration of 0.05 M gave optimum phosphate response. At lower concentrations the response continued to increase, whereas at higher concentrations the response gradually decreased. Higher buffering capacity of the buffer at higher concentrations affects phosphate response due to its high ionic strength, which in turn hinders the movement of H_2O_2 to the electrode (Adeloju and Moline, 2001).

Effect of pyrrole concentration: Figure 5 shows the influence of pyrrole concentration on the amperometric response for phosphate. The optimum response was

obtained when the film was formed in 0.3 M pyrrole. At lower concentrations, the sensitivity of the response was low, possibly due to inadequate coverage of the electrode and insufficient entrapment of phosphate. Beyond 0.3 M pyrrole, the response decreased slightly and had a noisy background. The film appeared darker and thicker as well. This could be due to an increase in the thickness of the PPy-PNP-XOD-$Fe(CN)_6^{4-}$ film at the higher pyrrole concentrations. The decrease in sensitivity could be due to an increase in the diffusion barrier caused by the thicker film.

Effect of applied potential: Figure 6 shows the amperometric responses obtained for phosphate with the variation of the applied electrode potential between 0 and +800 mV. The response increased as the applied potential became less positive, reaching an optimum value at +200 mV (vs. Ag/AgCl). A potential of +650 mV or -650 mV was applied by Gulbault (1984), while Konisita *et al.* (1995) applied +450 mV, D'Urso *et al.* (1990) applied +650 mV, Male and Luong (1991) applied +700 mV and Kulys *et al.* (1992) chose an optimum value at +200 mV (vs. Ag/AgCl). A potential of +650 mV or -650 mV was applied by Gulbault *et al.* (1995), while Konisita applied +450 mV, D'Urso applied +650 mV, Male and Luong applied +700 mV and Kulys *et al.* (1992) chose an optimum potential of +100 mV. The phosphate response obtained with an applied potential of +200 mV in the present study had a lower background and was more reproducible than at other applied potentials. At potentials that were lower than +200 mV, the phosphate responses were slightly lower and the background was very noisy. This may be due to the increased tendency to reduce oxygen at the negative potentials (Guilbault and Nanjo, 1975) as illustrated by equation (1 and 2). The reduction of the oxygen may have impaired the efficiency of the PPy-PNP-XOD-Fe $(CN)_6^{4-}$ film by suppressing the response. At potentials between +200 mV to +700 mV, the sensitivity of the response decreased considerably. And a possible reason for the biosensor instability and poor response may be due to the over-oxidation of the polymer at the higher positive potentials by Haemmerli *et al.* (1990).

It has been reported by Haemmerli *et al.* (1990) that at these potentials there may be a significant decrease in conductivity of the polymer film and, hence, a dramatic decrease in the sensitivity of the biosensor, which is associated with the over-oxidation of the polymer.

Analytical characteristics: A typical calibration curve obtained for phosphate on the PNP-XOD-$Fe(CN)_6^{4-}$ electrode is shown in Fig. 7. The amperometric response was linear for phosphate concentrations between 0.1 and

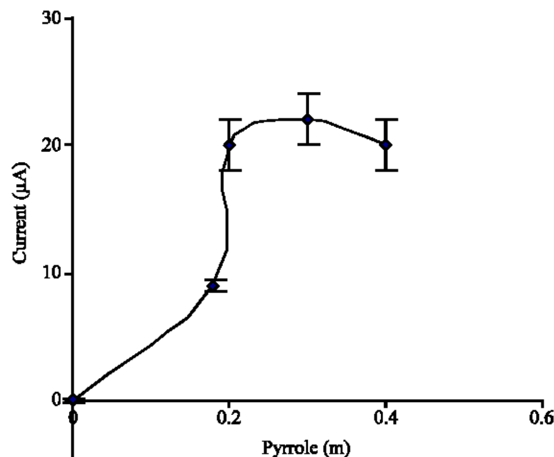


Fig. 5: Effect of pyrrole concentration on phosphate response obtained with PPy-PNP-XOD-Fe $(CN)_6^{4-}$ electrode. [Phosphate] was 10 mM. The monomer solution contained 6.2 U mL^{-1} of XOD, current density: 0.5 mA cm^{-2} polymerisation period: 200 sec 50 mM $Fe(CN)_6^{4-}$ and E_{app} 200 mV

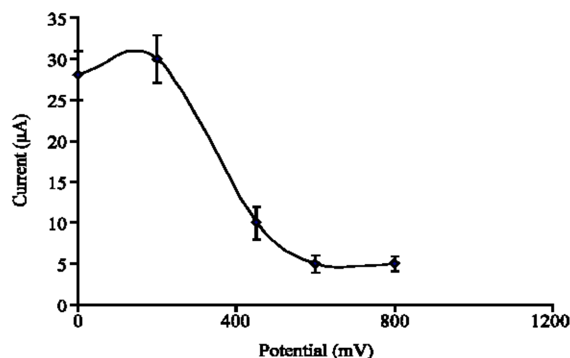


Fig. 6: Effect of applied potential on phosphate response obtained with PPy-PNP-XOD-Fe $(CN)_6^{4-}$ electrode. [Phosphate] was 10 mM. The monomer solution contained 0.3 M pyrrole, 6.2 U mL^{-1} of XOD, current density: 0.5 mA cm^{-2} polymerisation period: 200 sec and 40 mM $Fe(CN)_6^{4-}$

1 mM. The minimum detectable phosphate concentration with the biosensor was 1.0 mg L^{-1}, while Ikebukuro *et al.* (1995) also reported a detection limit of 0.3 mg L^{-1} Minimum detectable concentration was determined by the lowest phosphate concentration that gives a response. However the sensitivity of the biosensor is not yet sufficient to detect phosphate in river water because an ANZECC maximum permissible concentration is 0.46 mg L^{-1} (ANZECC, 2002).

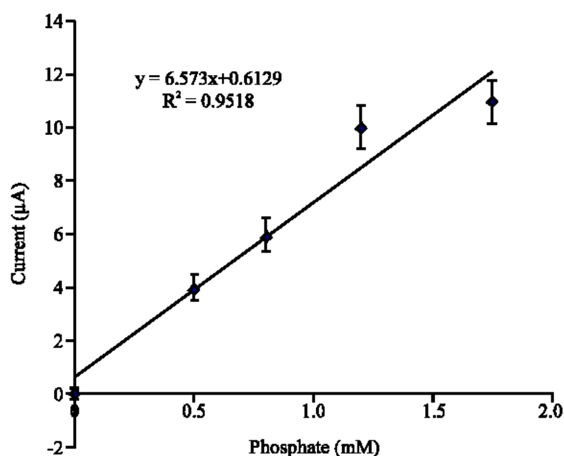


Fig. 7: Typical calibration graph obtained for phosphate with the PPy-PNP-XOD-$Fe(CN)_6^{4-}$ electrode. The monomer solution contained 0.3 M pyrrole, 6.2 U mL^{-1} of XOD, current density: 0.5 mA cm^{-2} polymerisation period: 200 sec 40 mM $Fe(CN)_6^{4-}$ and E_{app}.200 mV

Stability of biosensor response: Phosphate response obtained with the PPy-PNP-XOD-$Fe(CN)_6^{4-}$ decreased slowly by 20% of its initial value after 24 h. In general, the PPy-PNP-XOD-$Fe(CN)_6^{4-}$ electrode lost more than 20% of its sensitivity per day if it was not stored in barbitone buffer at pH between 7 and 7.8. The sensitivity of the biosensor decreased rapidly for the first 2 days and at 8th day it had reached 50% of its initial value. Beyond 12 days it continued to decrease and eventually stabilised at about 20% of its initial value after 2 weeks. The observed decrease in sensitivity of the biosensor may be attributed to loss of enzyme and ferrocyanide into the bulk solution. Male and Luong (1991) have reported that the use of the same bienzyme system on reactivated nylon membrane result in a loss of 30% of its response in three weeks.

The lower loss observed in this case may be attributed to its use in the FIA mode where the electrode was only in direct contact with the analyte for a relatively short period. Despite the 20% decrease in sensitivity observed in the present study, the resulting response is still useful for quantification of phosphate by standard addition methods. However, when the PPy-PNP-XOD-$Fe(CN)_6^{4-}$ electrode is stored in a barbitone buffer solution, the stability of enzymes is improved, optimum sensitivity is attained and analysis of phosphate ion is improved. D'Urso and Coulet (1990) and Guilbault and Nanjo (1975) also used barbitone as buffer in preference to other buffers while they analyse phosphate. Other buffer solutions investigated in this study (citrate, borate and tris-HCl buffer) gave poor responses for phosphate ions and might have had adverse effect on the activities of the enzymes. Guilbault and Nanjo (1975) have reported that borate buffer inhibited enzyme in the same way as phosphate ions.

CONCLUSION

Galvanostatic polymerisation has been successfully used to immobilise XOD, PNP and potassium ferrocyanide in polypyrrole and has enabled fabrication of an amperometric phosphate biosensor. The optimum conditions achieved for the formation of PPy-PNP-XOD-$Fe(CN)_6^{4-}$ include 0.5 M of pyrrole, 6.2 U mL^{-1} of XOD, 42.2 U mL^{-1} of PNP, 50 mM of $K_4Fe(CN)_6$, a polymerisation time of 200 sec and an applied current density of 0.5 mA cm^{-2}. The optimum applied potential for amperometric detection of phosphate was +200 mV vs. Ag/AgCl (3M KCl). The minimum detectable phosphate concentration under these conditions was 0.01 mM or 10 µM and the linear concentration range was between 0.1 and 1 mM. The biosensor could be used for more than 1 week for bioprocess and environmental monitoring with out any significant drop in sensitivity.

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