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Polypyrrole Based Amperometric and Potentiometric Phosphate Biosensors: A Comparative Study

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Abstract: The preparation of two electrochemical (potentiometric and amperometric) phosphate biosensors is described and compared. Purine Nucleoside Phosphorylase (PNP) and xanthine oxidase (XOD) were co-immobilised via entrapment into polypyrrole (PPy) films by galvanostatic polymerization. Polypyrrole entrapment was achieved with 0.5 M pyrrole by using a polymerisation time of 200 sec and a mole ratio of 1:8 (6.2 U mL^{-1} XOD: 49.6 U mL^{-1} PNP) in both biosensors. Sensitive amperometric measurements were compared with those of potentiometric measurements obtained for PPy-PNP-XOD-$Fe(CN)_6^{4-}$ biosensors. A minimum detectable concentration of 1.0 μM phosphate and a linear concentration range of 5-20 μM were achieved with potentiometric PPy-PNP-XOD-$Fe(CN)_6^{4-}$ biosensor. In comparison, a minimum detectable concentration of 10 μM and a linear concentration range of 0.1-1 mM were achieved with amperometric biosensor. The presence of uric and ascorbic acids had the least effect on the performance of the amperometric and potentiometric PPy-PNP-XOD-$Fe(CN)_6^{4-}$ biosensors, therefore will not have any effect on phosphate measurement in both biosensors at levels normally present in water.

Key words: Amperometric detection, polypyrrole, potentiometric detection, phosphate, biosensor

INTRODUCTION

Phosphate's widespread presence in detergent and fertiliser has made its determination of great importance to environmental pollution control. High phosphate concentration can pollute water resources and causes eutrophication of lakes and rivers (Nakamura, 2010). The eutrophication of water by phosphate can lead to over-growth of plant and toxic algae, thereby making it unsuitable for drinking or industrial use. In food analysis, the adverse effect of excess intake of phosphate in food additives upon human health is now a major consideration and is of vital interest to researchers (Nordin, 1997). Phosphate determination is also important in clinical diagnosis; the determination of phosphate in body fluid provides useful information about certain diseases and about the energetic state of cells and bone function (Kivlehan *et al*., 2009; Shervedani and Pourbeyram, 2009).

Phosphate determination based on spectroscopy (Galhardo and Masini, 2000; Mckelvie, 2000; Li *et al*., 2002; Nakamura, 2004; Yaqoob *et al*., 2004; Lin *et al*., 2006; Okoh *et al*., 2006; Gimbert *et al*., 2007; Nevesa *et al*., 2008) and chromatographic techniques (Galceran *et al*., 1993; Bello and Gonzalez, 1996; Colina *et al*., 1996; Zhu *et al*., 2008) were the analytical techniques commonly used for phosphate site monitoring. However, these required the use of hazardous chemicals for sample pre-treatment which are time consuming. They also produced toxic waste, such as tartaric acid and heavy metal such as antimony. Ion selective electrodes were also used for phosphate determination, based on various metals and associated complexes such as Sn complexes (Chaniotakis *et al*., 1993; Sasaki *et al*., 2004), hydroxyapatite (Petrucelli *et al*., 1996) cobalt metallic wires (Chen *et al*., 1997, 1998; De Marco and Phan, 2003; Gimbert *et al*., 2007; Bai *et al*., 2011). Comparatively these electrodes suffered from low selectivity and poor stability of the ion selective membranes. Other methods used for determination of phosphate included flow injection systems based on luminescence (Kyung and Hoon, 2009; Andolina and Morrow, 2011; Cardemil *et al*., 2010), chemiluminescence (Kawasaki *et al*., 1989; Nakamura *et al*., 1999; Nakamuraac *et al*., 2003; Yaqoob *et al*., 2004; Motomisu and Li, 2005) and florescence reactions (Motomisu and Li, 2005; Wang *et al*., 2010), conductometry (Zhang *et al*., 2008) and screen printed electrodes (Kwan *et al*., 2005; De Albuquerque and Ferreira, 2007; Zou *et al*., 2007; Khaled *et al*., 2008).

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A simple alternative is the use of an enzyme sensor that is able to measure phosphate directly in the sample. Enzyme sensors 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 enzyme sensors are phosphate biosensors that use, as biorecognition elements, substances such as nucleoside phosphorylase and xanthine oxidase (D'Urso and Coulet, 1990, 1993; Male and Luong, 1991; Wollenberger *et al*., 1992; Su and Mascini, 1995; Chen *et al*., 1997; Vazquez *et al*., 2003) and most commonly used alkaline phosphatase (Adeloju and Lawal, 2011). Others are acid phosphatase (Guilbault and Nanjo, 1975; Guilbault, 1984), glucose oxidase (Su and Mascini, 1995; Zhang *et al*., 2008), pyruvate oxidase (Mori and Kogure, 1994; Ikebukuroa *et al*., 1996; Mak *et al*., 2003; Roger *et al*., 2005; Rahman *et al*., 2006; Akylmaz and Yorgancia, 2007), sucrose phosphorylase (Adeloju and Lawal, 2011), phosphoglucomutase and glucose 6-phosphate dehydrogenase (Adeloju and Lawal, 2011). Combination of Maltose Phosphorylase (MP), mutarotase (MR) and glucose oxidase (GOX) have recently been used for fabrication of phosphate biosensors (Mousty *et al*., 2001; Zhang *et al*., 2008).

Electrochemical biosensor has made the production of screen printed electrode easier and amenable to miniaturisation of biosensors (Roger *et al*., 2005; De Albuquerque and Ferreira, 2007; Zou *et al*., 2007; Khaled *et al*., 2008). This has facilitated its mass production for quick and quantitative phosphate site monitoring or evaluation.

Amperometric biosensor requires three electrodes and application of potential before measurable current can be obtained. The use of enzyme-based amperometric biosensor has increased considerably in the past ten years as a result of its high selectivity and the sensitivity of amperometric signal (Mak *et al*., 2003; De Albuquerque and Ferreira, 2007; Lawal and Adeloju, 2009a). There are few potentiometric biosensors that require simple construction of two electrodes and without the application of potentials for measurable potential signal to be generated (Adeloju and Lawal, 2005; Lawal and Adeloju, 2009a; Adeloju and Lawal, 2011). Amperometric sensing can introduce interference as a result of oxidation of other matrix components which can lead to erroneous and enhanced current signal. However, in potentiometric sensing, oxidation of other matrix components are avoided). Villalba *et al*. (2009) recently reviewed the advantages and disadvantages of the electrochemical biosensors for the determination of phosphate.

In the PNP-XOD bienzyme system employed recently in various studies (Cosnier *et al*., 1998; Adeloju and Lawal, 2005; Lawal and Adeloju, 2009b; Lawal and Adeloju, 2009a; Adeloju and Lawal, 2011) showed a higher amount of hypoxanthine was produced during enzymatic phosphate recycling. Enzymatic phosphate recycling also took place using MP/GOX/Ap trienzyme for low level phosphate detection (Conrath *et al*., 1995; Huwel *et al*., 1997; Mousty *et al*., 2001). Wollenberger (Wollenberger *et al*., 1992) employed amplification by enzymatic substrate recycling in order to lower the detection limit, involving co-immobilisation of alkaline phosphatase (aP) and glucose oxidase. In the presence of phosphate ion inosine was phosphorylated by PNP to ribose-1-phosphate. Phosphate was then liberated by aP catalysis and became available again for inosine phosphorylation. Phosphate was thus recycled between aP and PNP while a higher amount of hypoxanthine was produced and recognised by sequential oxidation by XOD Hypoxanthine was subsequently oxidised to H_2O_2 catalysed by XOD (Watanabe *et al*., 1987; D'Urso and Coulet, 1990, 1993; Wollenberger *et al*., 1992), as given in Eq. 1 and 2.

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

and:

$$\text{Hypoxanthine+2H}_2\text{O+2O}_2 \xrightarrow{\text{PNP}} \text{Uric acid+2H}_2\text{O}_2 \quad (2)$$

In this study, biosensors were developed based on the enzymatic reaction shown in Eq. 1 and 2.

The successful immobilisation of XOD into polypyrrole film by Lawal and Adeloju (2009a, b, 2010) demonstrates that co-immobilisation with PNP is feasible. To this end, the comparison of electrochemical phosphate PPy-PNP-XOD biosensors in current study involved investigations of the effect of enzyme (PNP:XOD) mole ratio, pH, inosine and buffer concentrations, analytical characteristic, stability and common interferences affecting the two electrochemical biosensors.

MATERIALS AND METHODS

Reagents and standard solutions: Xanthine oxidase (XOD) (EC.1.2.3.2.2 Gradel (2.0 U mg^{-1}) from buttermilk, purine nucleoside phosphorylase (PNP) (EC.2.4.2.1.2 (15 U mg^{-1}), potassium ferrocyanide and pyrrole were obtained from Sigma. Pyrrole was distilled under vacuum at 130°C prior to use and this was stored in an aluminium foil-covered sample bottle in the freezer to prevent UV

degradation until required for use. $K_4Fe(CN)_6$ also undergoes UV degradation and so the solution was stored until required. XOD stock was stored in the refrigerator and PNP was stored in the freezer until required. All chemicals used were reagent grade and all compounds used in this work were prepared without further purification.

Instrumentation: A potentiostat/galvanostat designed and constructed in our laboratories was used for electrochemical measurements the potentiostat/ galvanostat was used in galvanostatic mode for the electropolymerisation. A three-electrode cell, which contained platinum working electrode, a platinum wire counter electrode and a Ag/AgCl (3 M KCl), was employed for amperometric detection of phosphate while potentiometric measurements were performed in a two electrode cell. The potentiostat was connected to a computer controller system. Solution was stirred when necessary with a Sybron Thermolyne (model S-17410) stirrer.

Enzyme immobilisation

Electrode preparation: A 320 μm aluminium oxide powder was used to polish the platinum working electrode with a soft polishing pad, to remove any previous film and then finally polished with 5 μm aluminium oxide. The electrode surface was washed thoroughly with Milli-Q water, rinsed under a stream of acetone and finally rinsed thoroughly with Milli-Q to remove any of the remaining aluminium oxide. The electrode was dried with fibre-free tissue paper and fixed onto a retort stand for the next step.

Electropolymerisation of PPy-PNP-XOD film: A three-electrode cell, which contained platinum working electrode, a platinum wire counter electrode and an Ag/AgCl (3 M KCl), was employed for electropolymerisation of PPy film. PPy-PNP-XOD-$Fe(CN)_6^{4-}$ biosensor, was made by immobilisation of xanthine oxidase (6.2 U mL^{-1}), purine nucleoside phosphorylase (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. The polymer electrode formed after galvanostatic polymerisation, 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 electrochemical measurements.

Amperometric measurements: An Ag/AgCl (3 M KCl) reference electrode, a platinum wire auxiliary electrode and PPy-PNP-XOD-$Fe(CN)_6^{4-}$ working electrode were assembled into a 20 mL voltametric cell. 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.

Potentiometric measurements: The electrode was rinsed thoroughly with distilled water to remove any loosely bound enzyme after electropolymerisation. Phosphate measurement was performed by placing the electrode into a magnetically stirred 20 mL (0.05 M) barbitone buffer solution, which contained 0.1 M NaCl and 5 mM inosine. The resulting equilibrium potential versus Ag/AgCl electrode was then measured after each addition of standard phosphate solution in a two-electrode cell. The interference of uric acid, ascorbic acid and glycine on the potentiometric responses were tested by their addition into the cell prior to the potentiometric measurements.

RESULTS AND DISCUSSION

Response to phosphate: In amperometric biosensor, XOD in the presence of molecular oxygen oxidised Hx and produced hydrogen peroxide which was detected by PPy-PNP-XOD-$Fe(CN)_6^{4-}$ biosensor. The reduced mediator was simultaneously regenerated on the electrode surface giving an amperometric signal directly proportional to phosphate concentration. But with potentiometric biosensor, a potential sensing signal was developed and the potential difference developed may have originated from the redox couple of hydrogen peroxide produced. Figure 1 shows a typical amperometric response of the amperometric biosensor upon the successive addition of phosphate into electrochemical cell with stirring.

Effect of PNP and XOD ratio: The performance of biosensors for environmental monitoring in terms of detection limit, stability and calibration range were strictly dependent on the enzyme loading. Figure 2 shows the results obtained for varying the XOD:PNP ratio in the outer layer. Both biosensors show that the optimum response was obtained when a mole ratio between 1:8 and 1:10 of XOD:PNP was incorporated into the outer layer. This is very similar to the mole ratio reported by D'Urso

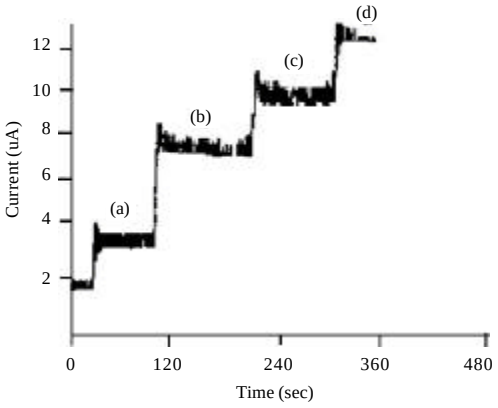

Fig. 1: Typical amperometric response of amperometric PPy-PNP-XOD-$Fe(CN)_6^{4-}$ biosensor to phosphate. (a) 10, (b) 20, (c) 30 and (d) 40 mM phosphate. The monomer solution contained 0.4 M pyrrole, current density: 0.5 mA cm^{-2} polymerisation period: 200 sec

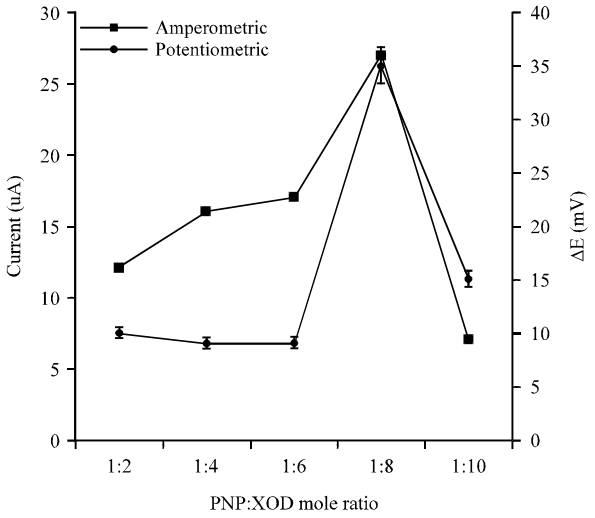

Fig. 2: Effect of immobilised (PNP) and (XOD) on phosphate response with both amperometric and potentiometric PPy-PNP-XOD-$Fe(CN)_6^{4-}$ biosensors. (XOD) was 6.2 U mL^{-1}, $Fe(CN)_6^{4-}$ was 50 mM and (Phosphate) was 10 mM. The monomer solution contained 0.4 M pyrrole, current density: 0.5 mA cm^{-2} polymerisation period: 200 sec

and Coulet (1990, 1993) for a phosphate biosensor. Guilbault and Cserfalvi (1976) reported a ratio of 1:10, while Kulys *et al.* (1992) and Wollenberger *et al.* (1992) reported a ratio of 1:5 for their construction of an amperometric phosphate biosensor. On the other hand

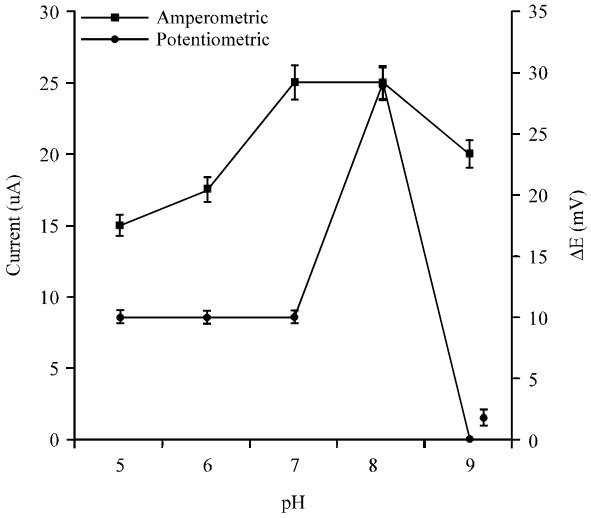

Fig. 3: Effect of pH on phosphate response obtained with both amperometric and potentiometric PPy-PNP-XOD- $Fe(CN)_6^{4-}$ biosensors. [Phosphate] was 10 mM. The monomer solution contained 0.4 M pyrrole, 6.2 U mL^{-1} of XOD, current density: 0.5 mA cm^{-2} polymerisation period: 200 sec and 50 mM $Fe(CN)_6^{4-}$

(Yao *et al.*, 2003) reported an optimum ratio of 1:21, while (Konisita *et al.*, 1995) found a ratio of 1:3 to be the optimum ratio for their amperometric phosphate biosensor.

Effects of pH and inosine concentration: Figure 3 shows the effect of pH on the response of phosphate on both amperometric and potentiometric PPy-PNP-XOD biosensors. The optimum pH of the sensors was found to be between 7 and 8 close to the optima of the two enzymes in solution (Berger and Grass, 1989; Guilbault and Nanjo, 1975; Kulys *et al.*, 1992) found that pH 7 produced the optimum sensitivity for an amperometric phosphate biosensor. While D'Urso and Coutlet (1990) and Yao *et al.* (2003) found pH 7.5 to be ideal for their amperometric phosphate biosensor. No response to 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. Wollenberger used excess inosine to ensure a co-reactant independent of phosphate response was obtained. Figure 4 illustrates the influence of inosine concentration on the phosphate response in both amperometric and potentiometric biosensors. The chosen inosine concentration of 10 mM was optimum for

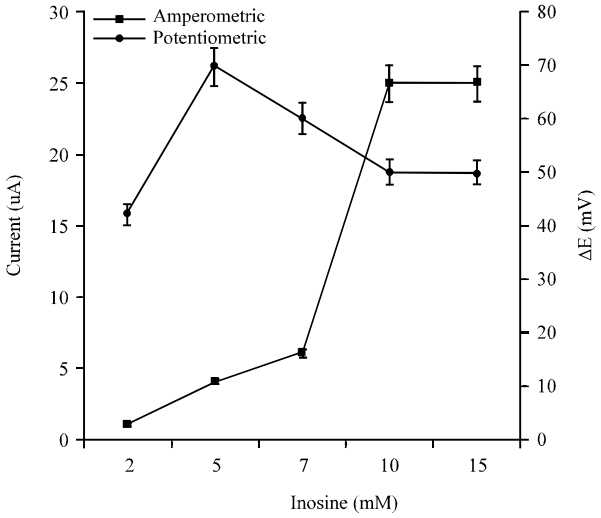

Fig. 4: Effect of inosine concentration on phosphate response obtained with both amperometric and potentiometric PPy-PNP-XOD- $Fe(CN)_6^{4-}$ biosensors. (Phosphate) was 10 mM. The monomer solution contained 0.4 M pyrrole, 6.2 U mL^{-1} of XOD, current density: 0.5 mA cm^{-2} polymerisation period: 200 sec and 50 mM $Fe(CN)_6^{4-}$

obtaining the most sensitive current response for phosphate in amperometric biosensor. Also further increase in inosine concentration did not raise the sensitivity of the phosphate response. For potentiometric biosensor, on the other hand 5 mM inosine was found to be sufficient. D'Urso and Coulet used 0.1 mM of inosine, while Used 5 mM of inosine in 0.05 M borate buffer. Wollenberger used 0.09 mom of inosine. Although, the amount of inosine used by Watanabe was not specified, it was in excess in 0.05 M Tris-HCl buffer solution. Guilbault 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 amperometric biosensor was higher, possibly due to the application of high voltage that could degrade inosine that lowers the electrochemical response. The amount used here was still close to the levels used in some studies.

Effect of buffer concentration: H_2O_2 and uric acid were produced when PNP catalysed the phosphorylysis of inosine in presence of phosphate to ribose-1-phosphate and hypoxanthine. The H_2O_2 and uric acid were electrochemically active and therefore could be easily detected. A signal could be obtained due to change in pH caused by the production of uric acid and optimisation of the buffer concentration was therefore essential. The influence of the concentration of buffer solution on the biosensors response is shown in Fig. 5.

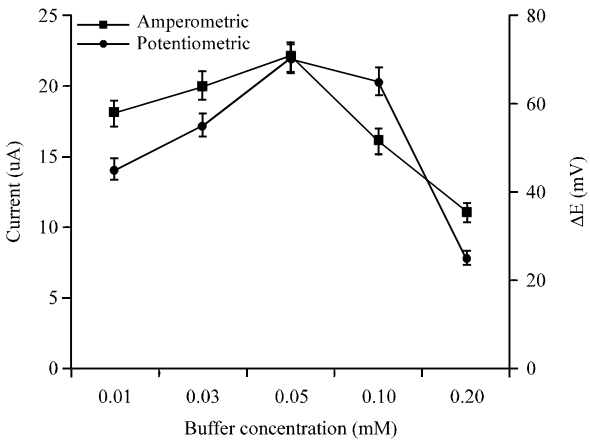

Fig. 5: Effect of buffer concentration on phosphate response obtained with both amperometric and potentiometric _PPy-PNP-XOD-Fe $(CN)_6^{4-}$ biosensors. (Phosphate) was 10 mM. The monomer solution contained 0.4 M pyrrole, 6.2 U mL^{-1} of XOD, current density: 0.5 mA cm^{-2} polymerisation period: 200 sec and 50 mM $Fe(CN)_6^{4-}$

In amperometric biosensor a buffer concentration of 0.05 M produced optimum phosphate response. At lower concentrations the current continued to increase, whereas at higher concentrations the current gradually decreased. Higher buffering capacity at higher concentrations of buffer due to its high ionic strength affected phosphate current response; this in turn hindered the movement of H_2O_2 to the electrode. While in potentiometric biosensor the increasing buffer concentration initially reduced the potentiometric response and then increased with decreasing buffer concentration up to 0.05 M as in amperometric biosensor. A weak buffer was therefore necessary to enable adequate measurement of the potentiometric response, because the higher buffering capacity of the more concentrated buffer solution affected the magnitude of the response. The optimum buffer concentration of 0.05 M was used for both amperometric and potentiometric biosensors.

Interference characteristics: Ascorbic Acid (AA) is considered to be a major interferant to most biosensors because of its relatively high concentrations in biological samples and low oxidation potential (Vidal *et al*., 2000). Figure 5 shows that the presence of ascorbic acid of up to 1 mM did not interfere with the phosphate response. However, the data shows that the addition of 5 mM ascorbic acid resulted in the enhancement of the phosphate response by 28%. The effect of AA at higher concentration on the response is even more dramatic. This enhancement effect of AA is very similar to those

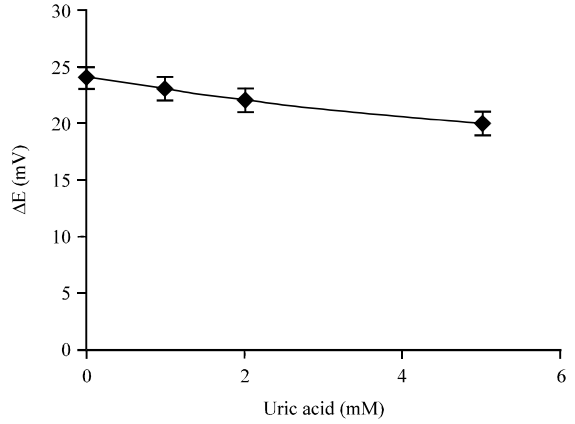

Fig. 6: Effect of uric acid on the potentiometric response of phosphate obtained with BSA-GLA-XOD-PNP biosensor. Potentiometric measurement was made in 0.05 M tris -HCl buffer. (Phosphate) was 10 mM. XOD:PNP ratio was 1:8

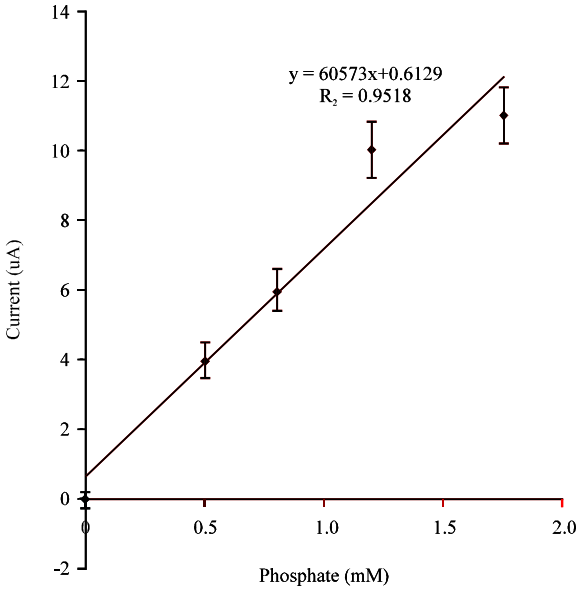

Fig. 7: Typical calibration graph obtained for phosphate with amperometric PPy-PNP-XOD-Fe(CN)$_6^{4-}$ biosensor. Phosphate was 10 mM. The monomer solution contained 0.4 M pyrrole, 6.2 U mL^{-1} of XOD, current density: 0.5 mA cm^{-2} polymerisation period: 200 sec, 200 mV and 50 mM Fe(CN)$_6^{4-}$E$_{app}$.200 mV

reported for other phosphate and glucose biosensors that employ the same enzyme immobilisation method (Su and Mascini, 1995; Adeloju and Moline, 2001) However, it is unlikely that the presence of low concentrations of ascorbic acid in water will affect the performance of the phosphate biosensor when used for analysis of water samples. AA concentration greater than 2.5 mM enhanced the response by up to 42%.

Another common interferant is uric acid but it is not as limiting as ascorbic acid (Su and Mascini, 1995; Adeloju and Moline, 2001). The presence of 1 mM uric acid suppressed the potentiometric response of the biosensor by 4%. It has been found in previous study (Kulys *et al*., 1992) that the sensitivity of the enzyme-based biosensors is degraded by the presence of uric acid. Smooth baselines and well defined responses were obtained for concentrations of uric acid less than 1 mM. When PPy-PNP-XOD-Fe(CN)$_6^{4-}$ biosensor fabricated by enzyme immobilisation in polypyrrole film was used, the presence of 2.5 mM uric acid the change in potential was suppressed by 17% as shown in Fig. 6. The presence of a high concentration of uric acid in water or other aqueous solutions favours the oxidation of uric acid with hydrogen peroxide, thus suppressing the electrochemical response of the phosphate. Interference of AA with phosphate sensor may be due to oxidation of ascorbic acid by the H_2O_2 produced from the enzymatic reaction. Adeloju and Moline (Adeloju and Moline, 2001) also found that ascorbic acid and uric acid affected their potentiometric PPy film sensor. The presence of glycine in water or other aqueous solutions did not interfere with both biosensors' response to phosphate and it is probable that other proteins would not interfere either. However, the performance of both PPy-PNP-XOD-Fe(CN)$_6^{4-}$ biosensors fabricated by enzyme immobilisation in polypyrrole film for the determination of phosphate in water samples will not be affected by the presence of low concentrations of uric acid.

Analytical characteristics: Figure 7 shows a typical calibration curve obtained for amperometric phosphate biosensor (PNP-XOD-Fe(CN)$_6^{4-}$. The amperometric response was linear for phosphate concentrations between 0.1 and 1 mM. The minimum detectable phosphate concentration with the biosensor was 10 μM. (1.0 mg) On the other hand, the calibration curve obtained for potentiometric biosensor is as shown in Fig. 8. The potentiometric response was linear between 5 and 25 μM (0.5-2.5 mg L^{-1}) and the minimum detectable phosphate concentration was 1 μM (0.1 mg L^{-1}) which is much lower than the minimum obtained in amperometric biosensor. It is also lower than the limit of detection for phosphate using AP/GOX combination (4 μM) (Villalba *et al*., 2009), pyruvate biosensor of Kwan-Roger (3.6 μM) (Roger *et al*., 2005) and is also comparable to those obtained with photometric molybdite complex (0.3 μM) (Villalba *et al*., 2009) or sensitive fluorescence methods (0.8 μM) (Villamil-Ramos and Yatsimirsky, 2011).

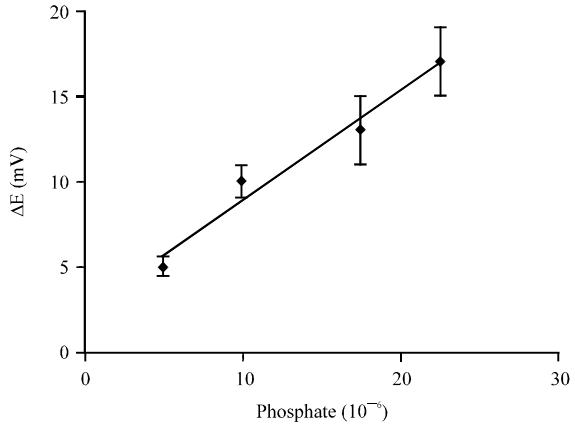

Fig. 8: Typical calibration graph obtained for phosphate with potentiometric PPy-PNP-XOD-$Fe(CN)_6^{4-}$ biosensor. (Phosphate) was 10 mM. The monomer solution contained 0.4 M pyrrole, 6.2 U mL^{-1} of XOD, current density: 0.5 mA cm^{-2} polymerisation period: 200 sec and 50 mM $Fe(CN)_6^{4-}$

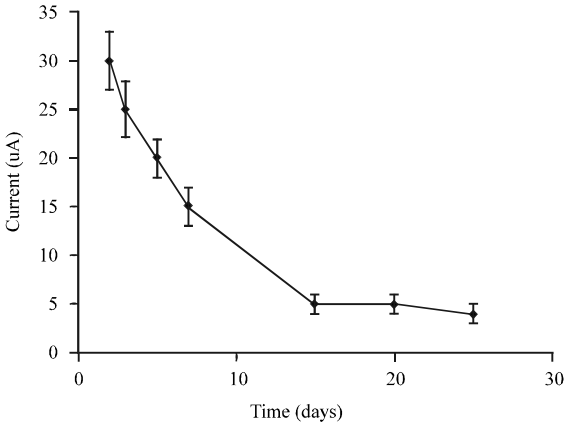

Fig. 9: Influence of storage time on the sensitivity of phosphate response obtained with amperometric PPy-PNP-XOD-$Fe(CN)_6^{4-}$ biosensor. Amperometric measurement was performed under the following conditions: 0.05 M barbitone buffer, applied potential of 200 mV and 10 mM inosine. Phosphate was 10 mM

Potentiometric biosensor has the same minimum detection limit (0.1 μM) as that of conductometry biosensor produced by Zhang *et al.* (2008). This is enough to probe phosphate concentration within inland water ways. A plot of the potential versus logarithm of phosphate concentration produced a slope of 46.5±1.0 mV/decade which was higher than the expected 29.5 mV/decade for a two-electron process. However, this value was similar to a slope of 45.5 mV/decade reported previously by Menzel for a phosphate biosensor (Menzel and Lerch, 1995). The observed linear relationship between changes in potential and phosphate concentration, instead of log concentration, may be due to the complex nature of the composite electrode and the extent to which the Nernstian behaviour is maintained, which is also dependent on various parameters, such as enzyme loading, film thickness and substrate (phosphate ion). Further work is necessary to improve the sensitivity of these biosensors to permit direct determination of phosphate concentrations in water, as these are still not sensitive enough for phosphate analysis in potable water.

Stability of biosensor response: Figure 9 shows that the phosphate response obtained with the amperometric 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 amperometric response of the biosensor decreased rapidly for the first two days and at eighth 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 two weeks. In potentiometric biosensor, the response started to decrease after two weeks of continuous use. It maintained 80% of its initial potentiometric response in the first three weeks but after four weeks there was a decrease of 60% of it initial potentiometric response. The observed decrease in electrochemical responses of the biosensors may be attributed to the loss of enzyme and ferrocyanide into the bulk solution and also possibly be to lifetime of the enzymes.

In conductometry phosphate biosensor produced by Zhang *et al.* (2008), 70% of the initial response was lost after two weeks and 50% after three weeks. Male and Luong have reported that the use of the same bienzyme system on reactivated nylon membrane resulted in a loss of 30% of its response in three weeks (Male and Luong, 1991). The lower loss observed in Male and Louong (1991) study may be attributed to its use in the FIA mode, where the electrode was in direct contact with the analyte for an only relatively short period. Despite the 20% decrease in sensitivity observed in amperometric biosensor, 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.

CONCLUSIONS

Amperometric and potentiometric PPy-PNP-XOD-$Fe(CN)_6^{4-}$ biosensors have been fabricated for accurate potentiometric and amperometric measurements of phosphate at concentrations that are suitable for environmental monitoring. Potentiometric biosensor can detect a minimum of 1 μM phosphate ion and has a linear concentration range of 5-20 μM, where as amperometric biosensor can detect a minimum of 10 μM and has a linear range of 0.1-1 mM. Interferences from uric and ascorbic acids, at levels normally present, were not considered problematic for detection of phosphate with these biosensors. The biosensors appear promising for analysing phosphate in polluted water. However, further improvement in sensitivity was necessary before these biosensors are reliably used in unpolluted and portable waters.

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