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Comparison of enzyme immobilisation methods for potentiometric phosphate biosensors

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ABSTRACT

The development of two phosphate biosensors is described and compared for potentiometric detection of phosphate. Purine nucleoside phosphorylase (PNP) and xanthine oxidase (XOD) were co-immobilised by chemical cross-linking with glutaraldehyde (GLA) and bovine serum albumin (BSA), and via entrapment into polypyrrole (PPy) films by galvanostatic polymerisation. The BSA-GLA film was made with 4.5% v/v GLA and 6.8% w/v BSA with a drying time of 30 min, while polypyrrole entrapment was achieved with 0.5 M pyrrole by using a polymerisation time of 200 s. A mole ratio of 1:8 (6.2 U/mL XOD: 49.6 U/mL PNP) was used for both methods of enzyme immobilisation. Sensitive potentiometric measurements obtained for phosphate with the BSA-GLA-PNP-XOD biosensor were compared with those of PPy-PNP-XOD-Fe$(CN)_6^{4-}$ biosensor. A minimum detectable concentration of 0.1 mg/L phosphate and a linear concentration range of 0.5–2.5 mg/L were achieved with the PPy-PNP-XOD-Fe$(CN)_6^{4-}$ biosensor. In comparison, a minimum detectable concentration of 2 mg/L and a linear concentration range of 4–12 mg/L were achieved with the BSA-GLA immobilisation. The presence of uric and ascorbic acids had the least effect on the performance of the PPy-PNP-XOD-Fe$(CN)_6^{4-}$ biosensor, but will not have any effect on phosphate measurement with both biosensors at levels normally present in water.

1. Introduction

Various phosphate biosensors have been developed based on the use of an enzymatic sequence in which the first enzyme (usually a phosphorylase) uses phosphate as a co-substrate to generate a product which is a substrate for a second enzyme, usually an oxidase. Among these is a phosphate biosensor which uses a biorecognition element, a substance such as purine nucleoside phosphorylase (PNP) and xanthine oxidase (XOD).

Several methods of enzyme immobilisation have been employed for the electrochemical biosensing of phosphate. (Kulys et al., 1992; Wollemberger et al., 1992; D'Urso and Coulet, 1993; Mori et al., 1994; Conrath et al., 1995; Cosnier and Gondran, 1998; Mousty et al., 2001; Yao et al., 2003). Among these are enzyme immobilisation by adsorption (Yao et al., 2003), covalent bonding (Yao and Wasa, 1988), entrapment (Su and Mascini, 1995) and cross-linking (Konishita et al., 1995). In particular, the use of cross-linking has attracted the interest of many researchers due to its simplicity for direct immobilisation of enzymes on electrodes. Konisita et al. (Konishita et al., 1995) used glutaraldehyde (GLA) to immobilise XOD and PNP on a nylon membrane. Guilbault et al. (Guilbault and Nanjo, 1975) also immobilised XOD and PNP with GLA on a cellulose acetate membrane, while D'Urso et al. (D'Urso and Coulet, 1990; D'Urso and Coulet, 1993) used a mixture of bovine serum albumin (BSA) and GLA to immobilise XOD and PNP on a teflon membrane. Watanabe (Watanabe et al., 1987) used a mixture of GLA and BSA to immobilise XOD and PNP on a cellulose acetate membrane. Recently, Karakus et al. (Karakus et al., 2006) covalently attached pyruvate oxidase to nano conducting polymer layer on a glassy carbon electrode for amperometric detection of phosphate. Pyruvate oxidase was also used by other researchers (Gavalas and Chaniotakis, 2000; Roger et al., 2005; Rahman et al., 2006; Akyilmaz and Yorganci, 2007) for phosphate ion determination. Alkaline phosphatase (Tzanavarus and Themelis, 2002), acid phosphatase, maltose phosphorylase (Nakamura et al., 2003) and glucose oxidase (Su and Mascini, 1995), covalently attached to a cellulose nitrate membrane, were other enzymes used to determine phosphate. Rahman et al. (Rahman et al., 2006) developed a biosensor based on a conducting polymer (poly5,2′:5′.2′-terthiophene-3′-carboxylic acid) to modify pyruvate oxidase for phosphate ions determinations. A biosensor for inorganic phosphate based on the use of rhodamine labelled phosphate binding protein was developed by Okoh et al. (Okoh et al., 2006). Other methods, such as spectrophotometry based on molybdenum complex (Galhardo and Masini, 2000; Reza et al., 2003; Mas-Torres et al., 2004; Motomisu and Li, 2005; Nevesa et

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al., 2008), fluorescence (Gupta et al., 2005; Lin et al., 2006; Villalba et al., 2009), chemiluminesence (Nakamura et al., 1999; Nakamura et al., 2004; Yaqoob et al., 2004), screen printed electrodes (Roger et al., 2005; Khaled et al., 2008), conductometry (Zhiqiang et al., 2008) and flow injection analysis (FIA) methods (Fernández and Reis, 2002; De Marco and Phan, 2003; Diniz et al., 2004; Estela and Cerda, 2005; Gimbert et al., 2007) have recently been used to determine phosphate ion. The electrochemical biosensing methods described above have higher limit of detection for phosphate (>1 μM) than the spectroscopic methods (<0.01 μM). These are therefore not suitable for onsite testing of phosphate in water. However, the electrochemical biosensors are stable and specific as they possess fewer sources of electroactive interference that is inherent with the spectroscopic methods.

Most of these biosensors have been used for the detection of phosphate by amperometric monitoring of the oxidation current resulting from liberation of H_2O_2 or the reduction current of oxygen consumed during the enzymatic reaction. In contrast to amperometric detection, potentiometric measurement of phosphate requires construction of a simple phosphate biosensor based on the use of a two-electrode system. The enzyme, PNP, is known to catalyse the conversion of orthophosphate in the presence of inosine to hypoxanthine. In the PNP-XOD bienzyme system employed by various researchers (Watanabe et al., 1987; D'Urso and Coulet, 1993; Conrath et al., 1995; Cosnier and Gondran, 1998; Lawal and Adeloju, 2008) a higher amount of hypoxanthine was produced during enzymatic phosphate recycling. Hypoxanthine was subsequently oxidised to H_2O_2 catalysed by XOD (Watanabe et al., 1987; D'Urso and Coulet, 1990; Wollemberger et al., 1992; D'Urso and Coulet, 1993), as given by Eqs. (1) and (2).

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

and

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

In order to establish an efficient immobilisation strategy for the potentiometric biosensing of phosphate, we have compared two enzyme immobilisation methods: (a) the use of BSA and GLA for chemical cross-linking of PNP and XOD, and (b) entrapment of the enzymes into polypyrrole film. Important considerations for the chemical cross-linking of the enzymes will include the influence of drying time, PNP:XOD ratio, GLA and BSA concentrations. On the other hand, consideration for the galvanostatic immobilisation includes pyrrole concentration, applied current density, polymerisation time, pH and buffer concentration. Also, the suitability of the enzyme immobilisation methods for potentiometric biosensing of phosphate was further established by investigating the influence of ascorbic acid and uric acid on the sensitivity of the BSA-GLA-PNP-XOD and PPy-PNP-XOD-Fe$(CN)_6^{4-}$ biosensors to phosphate. The identification of the better approach for potentiometric measurement of phosphate will be made by comparison of the performances of these biosensors.

2. Materials and methods

2.1. Reagents, chemicals and standard solutions

All chemicals were of analytical reagent grade, unless specified otherwise. Pyrrole was supplied by Sigma–Aldrich, Sydney, Australia and was distilled before use. The distilled pyrrole was stored in the refrigerator under a nitrogen atmosphere in a container wrapped with aluminium foil to prevent UV degradation and air oxidation. All solutions were prepared with Milli-Q water. Xanthine oxidase (EC1.1.3.22 Grade 1; 2.0 U mg^{-1}) from buttermilk, purine nucleoside phosphorylase (EC2.4.2.1; 15 U mg^{-1}), inosine, and potassium ferrocyanide, were obtained from Sigma–Aldrich, Sydney, Australia. The GLA stock solution was deliberately lower than the BSA stock solution used in the mixture for formation of the layers to avoid excess cross-linking and, hence, denaturing of PNP and XOD. Stock of BSA solution (15% w/v) and GLA solution (10% v/v) were prepared and stored in the refrigerator at −4 °C. These were subsequently diluted to give appropriate concentrations. Other chemicals used were also of analytical reagent grade, and used as received. Phosphate stock solution (0.5 M) was stored in the refrigerator and was diluted when necessary to give the required standard concentration. Barbitone buffer (pH 7) was prepared by neutralising barbituric acid with sodium hydroxide.

2.2. Instrumentation

Electrochemical deposition of PPy films was performed with a three-electrode cell, comprising of an Ag/AgCl (3 M KCl) reference electrode, a platinum gauze auxiliary electrode and a 1.5 mm platinum disc-working electrode. Potentiostat/galvanostat designed and built within our laboratories was employed for the electropolymerisation of pyrrole, as well as for potentiometric measurements which 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.

2.3. Enzyme immobilisation

2.3.1. Preparation of BSA-GLA-PNP-XOD biosensor

A two-step procedure was used for the immobilisation of PNP and XOD on a platinum electrode which was polished with 0.3 μM alumina. 1 μL aliquot of the BSA-GLA mixture was initially applied to a 10 mm platinum electrode to form the first layer and was allowed to air dry (when the mixture had gelatinised and hardened). This BSA-GLA layer was formed to improve the adhesion of the BSA-GLA-PNP-XOD layer. Once the first layer has dried, 3 μL of the second mixture for layer 2, made up of 5 μL of 6.8% w/v of BSA, 5 μL of 4.5% v/v GLA, 6.2 U/mL XOD and 49.6 U/mL PNP, was then spread on top of the first layer. This layer was also left to dry, until the film had gelatinised and hardened. Prior to use for potentiometric measurements, the electrode was washed thoroughly under a stream of Milli-Q water to remove any loosely bound molecules. Optimum conditions for the BSA-GLA immobilisation were: 6.8% w/v BSA, 4.5% w/v GLA and air drying time of 30 min.

2.3.2. Preparation of PPy-PNP-XOD-Fe$(CN)_6^{4-}$ biosensor

Platinum disc electrodes were polished with 0.3 μm alumina on a polishing pad, rinsed with Milli-Q water, acetone and again with water. Before electropolymerisation, the pyrrole (0.1–0.5 M) solution was purged with nitrogen for about 10 min to remove dissolved oxygen. Potassium ferrocyanide, xanthine oxidase and purine nucleoside phosphorylase were immobilised into the polypyrrole film by electropolymerisation at various current densities and polymerisation times. Optimum conditions achieved for the PPy immobilisation are: 0.5 M pyrrole, polymerisation time of 200 s, and an applied current density of 0.75 mA/cm^2.

2.3.3. Potentiometric measurements

After electropolymerisation and entrapment into polypyrrole film or immobilisation into BSA-GLA layer, the electrode was rinsed thoroughly with Milli-Q water to remove any loosely bound enzyme. Phosphate measurement was performed by placing the electrode in a magnetically stirred 20 mL (0.05 M) barbitone buffer solution, which contained 0.1 M NaCl and 5 mM inosine. The

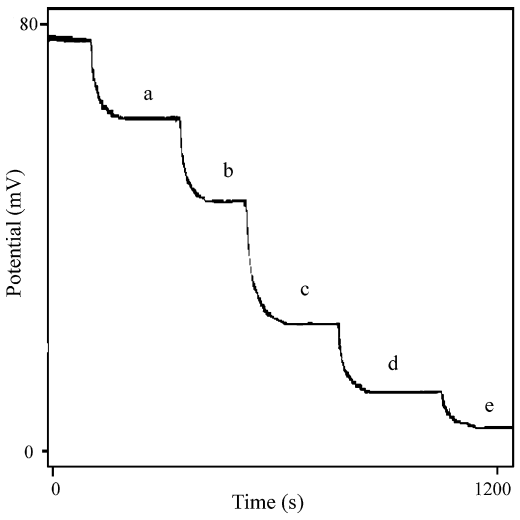

Fig. 1. Dependence of the potentiometric responses of PPy-PNP-XOD-Fe(CN)$_6^{4-}$, biosensor on phosphate concentration. (a) 10, (b) 20, (c) 30, (d) 40 and (e) 50 mM phosphate.

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 and ascorbic acid on the potentiometric responses were tested by addition into the cell prior to the potentiometric measurements.

3. Results and discussion

3.1. Potentiometric response of the biosensor to phosphate

The reactions involved in the potentiometric detection of phosphate with both phosphate biosensors are given by equations 1 and 2. PNP catalysed the phosphorylation of inosine to ribose-1-phosphate and Hx. XOD, in the presence of molecular oxygen, oxidised Hx and produced hydrogen peroxide which was detected. A potential difference developed due to the hydrogen peroxide produced and this was used for phosphate measurement. Fig. 1 shows the potentiometric response of the PPy-PNP-XOD-Fe(CN)$_6^{4-}$ biosensors to increasing phosphate concentration.

3.2. Effect of XOD:PNP ratio

The suitability of the biosensor for phosphate measurement in terms of detection limit, stability and linear concentration range are strictly dependent on the enzyme loading. Fig. 2 shows the results obtained for varying XOD:PNP ratio in the outer layer. Both biosensors show that the optimum phosphate response was obtained when a mole ratio between 1:8 and 1:10 of XOD:PNP was incorporated into the outer layer. This observation is similar to the mole ratio reported by D'Urso et al. (D'Urso and Coulet, 1990; D'Urso and Coulet, 1993). The phosphate potentiometric responses obtained with films formed with this XOD:PNP ratio, which is equivalent to 6.2 U/mL XOD and 49.6 U/mL PNP, were very reproducible with relative standard deviation (RSD) of 5.8% (n = 4) and the baselines were fairly smooth.

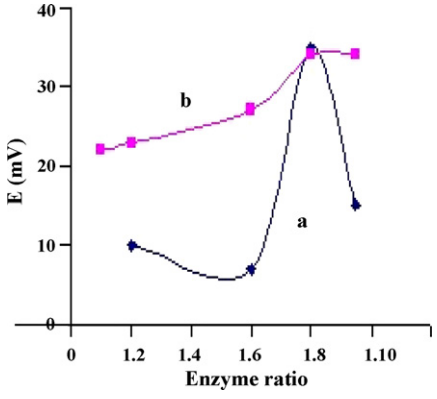

Fig. 2. Effect of varying PNP:XOD ratio on the potentiometric response of phosphate obtained with (a) PPy-PNP-XOD-Fe(CN)$_6^{4-}$ and (b) BSA-GLA-PNP-XOD biosensors. Measurement was made in 0.05 M Tris–HCl buffer. [Phosphate] was 10 mM and drying time was 30 min, n = 4.

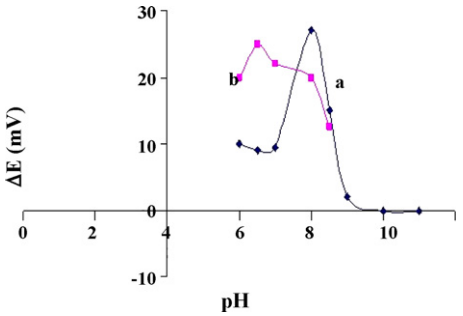

Fig. 3. Effect of varying pH on the potentiometric response of phosphate obtained with (a) PPy-PNP-XOD-Fe(CN)$_6^{4-}$ and (b) BSA-GLA-PNP-XOD biosensors. Measurement was made in 0.05 M Tris–HCl buffer. [Phosphate] was 10 mM, XOD:PNP ratio was 1:8, BSA was 6.8% w/v, GLA was 4.5% v/v and drying time was 30 min, n = 4.

At mole ratios higher than 1:8–1:10, the response was not as sensitive, possibly due to the lower PNP concentration in the BSA-GLA film. On the other hand, at a higher PNP concentration, the response sensitivity seems to plateau indicating that optimum catalytic effect was obtained in the presence of 1:8–1:10 ratios of XOD:PNP and no further improvement was obtained with further addition of PNP. As shown in Fig. 2a the XOD:PNP ratio of 1:8 also gave optimum phosphate response with PPy-PNP-XOD-Fe(CN)$_6^{4-}$ biosensor fabricated by enzyme immobilisation in polypyrrole film. Notably, at higher ratio than 1:8, the response diminished considerably and did not plateau as for the BSA-GLA-PNP-XOD biosensor. The same XOD:PNP mole ratio of 1:8 was therefore used for both methods of immobilisation.

3.3. Effect of pH and buffer concentration

The pH of the buffer used for measuring phosphate was varied and the resulting effects on sensitivity of the biosensors are illustrated in Fig. 3. The optimum phosphate response was obtained at pH 6.8 (Fig. 3b) with the BSA-GLA-PNP-XOD biosensor. This pH is in agreement with those reported previously (Haemmerli et al., 1990; Male and Luong, 1991; Kulys et al., 1992; D'Urso and Coulet, 1993) as optimum for the catalytic activity of PNP and XOD in phosphorylation of inosine and the oxidation of hypoxanthine to H_2O_2 and uric acid. Immobilisation of the enzymes in BSA-GLA mixture may be responsible for this lower pH obtained with Tris–HCl buffer. On the other hand, an optimum pH of 7.8 was obtained with PPy-PNP-XOD-Fe(CN)$_6^{4-}$ biosensor, as shown in Fig. 3a. This pH is also in agreement with those reported previously by other researchers (Su and Mascini, 1995; Govender, 2001)

An increase in buffer concentration resulted in a decrease in the phosphate potentiometric response. The higher buffering capacity of a more concentrated buffer solution hindered the effect of the catalytic process on the biosensor and reduced the potentiometric response. The high ionic strength, which results from the use of higher buffer concentrations, affects the movement of H_2O_2 to the electrode surface. Adeloju and Moline (Adeloju and Moline, 2001) also observed reduction in potentiometric response at higher buffer

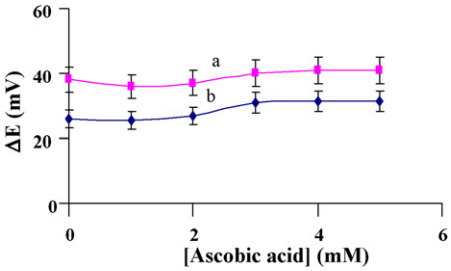

Fig. 4. Effect of ascorbic acid on the potentiometric response of phosphate obtained with (a) PPy-PNP-XOD-Fe(CN)$_6^{4-}$ and (b) BSA-GLA-PNP-XOD biosensors. Measurement was made in 0.05 M Tris–HCl buffer. [Phosphate] was 10 mM, XOD:PNP ratio was 1:8, BSA was 6 .8% w/v, GLA was 4.5% v/v, and drying time was 30 min, $n = 4$.

concentration with a glucose biosensor. The optimum potentiometric response was obtained with a buffer concentration of 12 mM with the BSA-GLA biosensor, while 5 mM buffer was optimum with the PPy-PNP-XOD-Fe(CN)$_6^{4-}$ biosensor. However, the establishment of equilibrium potential took longer time when 12 mM buffer was used. The observed benefit of the lower buffer concentration for the PPy-PNP-XOD-Fe(CN)$_6^{4-}$ biosensor may be due to the better porosity of its film compared with the BSA-GLA layer,

3.4. Interference study

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). Fig. 4 shows that the presence of ascorbic acid up to 1 mM did not interfere with the phosphate response. However, the data show that the addition of 5 mM ascorbic acid resulted in the enhancement of the phosphate response by 28%. The effect of AA on the response is even more dramatic at higher concentrations. This enhancement effect of AA is very similar to those 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. With PPy-PNP-XOD-Fe(CN)$_6^{4-}$ biosensor fabricated by enzyme immobilisation in polypyrrole film (Fig. 5b), 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 another study (Kulys et al., 1992) that the sensitivity of the enzyme-based

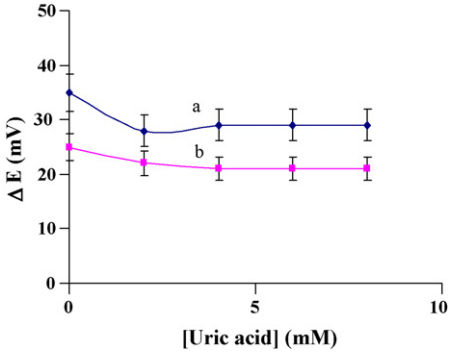

Fig. 5. Effect of uric acid on the potentiometric response of phosphate obtained, with (a) PPy-PNP-XOD-Fe(CN)$_6^{4-}$ and (b) BSA-GLA-PNP-XOD biosensors. Measurement was made in 0.05 M Tris–HCl buffer. [Phosphate] was 10 mM, XOD:PNP ratio was 1:8, BSA was 6 .8% w/v, GLA was 4.5% v/v, and drying time was 30 min, $n = 4$.

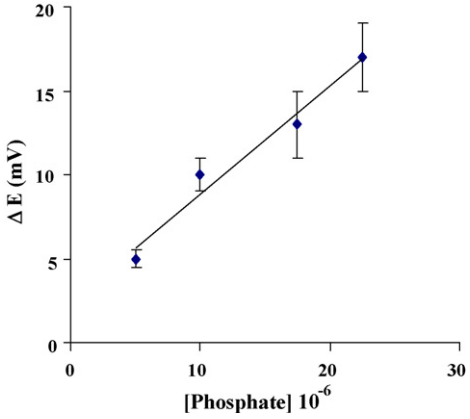

Fig. 6. Dependence of potentiometric responses of (a) PPy-PNP-XOD-Fe(CN)$_6^{4-}$ and (b) BSA-GLA-PNP-XOD biosensors on phosphate concentration. Measurement was made in 0.05 M Tris–HCl buffer. [Phosphate] was 10 mM, XOD:PNP ratio was 1:8, BSA was 6 .8% w/v, GLA was 4.5% v/v, and drying time was 30 min, $n = 4$.

biosensors is degraded by the presence of uric acid. Smooth baselines and well-defined responses were obtained in presence of uric acid less than 1 mM. With the PPy-PNP-XOD-Fe(CN)$_6^{4-}$ biosensor, the presence of 2.5 mM uric acid suppressed the potentiometric response by 17%, as shown in Fig. 5. 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 potentiometric response of phosphate. The interference of AA with phosphate biosensor 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 the presence of ascorbic acid and uric acid affected the potentiometric response of glucose obtained with a PPy-GOx biosensor. The presence of glycine did not interfere with phosphate measurements by both biosensors and it is probable that other proteins would also not interfere. However, the performance of both BSA-GLA-XOD-PNP and PPy-PNP-XOD-Fe(CN)$_6^{4-}$ biosensors for the determination of phosphate in water samples will not be affected by the presence of low concentrations of uric acid. Although, both methods were affected by the presence of higher ascorbic acid and uric acid concentrations, the resulting potentiometric response for phosphate was generally higher with the PPy-PNP-XOD-Fe(CN)$_6^{4-}$ biosensor than with the BSA-GLA-XOD-PNP biosensor.

3.5. Linear concentration range and minimum detectable concentration

The phosphate potentiometric response obtained with the PPy-PNP-XOD-Fe(CN)$_6^{4-}$ biosensor was linear from 5–25 μM (0.5–2.5 mg/L) as shown in Fig. 6. On the other hand, the phosphate response obtained with the BSA-GLA-XOD-PNP biosensor was only linear from 40 to 120 μM (4–12 mg/L) and then deviates from linearity between 100 and 200 μM, as shown in Fig. 7. A plot of the potential versus logarithm of phosphate concentration gave 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 et al. for a phosphate biosensor (Menzel et al., 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).

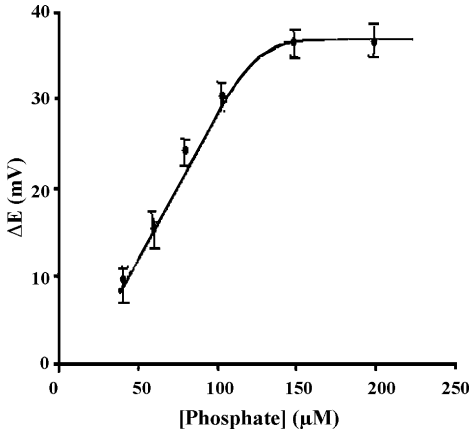

Fig. 7. Dependence of potentiometric response on phosphate concentration for BSA-GLA-PNP-XOD electrode. Potentiometric measurement was made in 0.05 M Tris–HCl buffer. [Phosphate] was 10 mM. XOD:PNP ratio was 1:8, BSA was 6 .8 %, GLA was 4.5 %, and drying time was 30 min, $n = 4$.

The minimum detectable concentration of phosphate achieved with the BSA-GLA-XOD-PNP biosensor was 2 mg/L, while the minimum detectable concentration obtained with the PPy-PNP-XOD-$Fe(CN)_6^{4-}$ biosensor was 0.1 mg/L. The detection limit achieved with the PPy-PNP-XOD-$Fe(CN)_6^{4-}$ biosensor was clearly much lower than achieved with the BSA-GLA-XOD-PNP biosensor, as well as with those reported by Ikebukwo and Wakamura (1996) and Menzel et al. (1995) that achieved 0.32 mg/L and 5 mg/L, respectively. Evidently, both biosensor fabrication methods considered in this study gave sensitive potentiometric responses for phosphate, but the PPy-PNP-XOD-$Fe(CN)_6^{4-}$ biosensor was much more sensitive than the BSA-GLA-XOD-PNP biosensor. However, both of these biosensors are still not sensitive enough for phosphate analysis in potable water. The minimum permissible concentration of phosphate in drinking water is 0.046 mg/L in Australia and New Zealand (ANZECC, 2002), while in Japan it is 0.33 mg/L (Nakamura et al., 1997). Further work is therefore necessary to improve the sensitivity of these biosensors to permit direct determination of phosphate concentrations in water.

4. Conclusions

A comparison has been made between a BSA-GLA-PNP-XOD phosphate biosensor, based on immobilisation of XOD and PNP (1:8) on a platinum electrode, with a PPy-PNP-XOD-$Fe(CN)_6^{4-}$ biosensor for potentiometric detection of phosphate. The BSA-GLA-PNP-XOD biosensor achieved a minimum detectable concentration of 2 mg/L, while the PPy-PNP-XOD-$Fe(CN)_6^{4-}$ biosensor gave a far more superior minimum detectable concentration of 0.1 mg/L. The achievable linear concentration range of 0.5–2.5 mg/L with the PPy-PNP-XOD-$Fe(CN)_6^{4-}$ biosensor was at the lower end of measurable concentrations, while that obtained with the BSA-GLA-PNP-XOD biosensor which ranged from 4–12 mg/L was clearly at the upper end. Interferences from uric and ascorbic acids, at levels normally present, were not considered to be a problem for potentiometric detection of phosphate with these biosensors, but the performance of the PPy-PNP-XOD-$Fe(CN)_6^{4-}$ biosensor was notably superior. However, there is still a need for further improvement in sensitivity to permit direct analysis of phosphate in pristine waters.

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