Biosensors and Bioelectronics 40 (2013) 377–384

Contents lists available at SciVerse ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Polypyrrole based amperometric and potentiometric phosphate biosensors: A comparative study B

Abdulazeez T. Lawal *, Samuel B. Adeloju

NanoScience and Sensor Technology Research Group, School of Applied Sciences and Engineering, Monash University, Churchill, Vic 3842, Australia

ARTICLE INFO

Available online 25 August 2012

Keywords:
Amperometric detection
Polypyrrole
Potentiometric detection
Phosphate
Biosensor

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-immobilized via entrapment into polypyrrole (PPy) films by galvanostatic polymerization. Polypyrrole entrapment was achieved with 0.5 M pyrrole by using a polymerization time of 200 s and a mole ratio of 1:8 (6.2 U/mL XOD: 49.6 U/mL PNP) in amperometric phosphate biosensor. Potentiometric bi-layer biosensor PPy–NO_3/BSA–GLA–PNP–XOD is made of an inner electropolymerized PPy–NO_3 layer and an outer layer of PNP and XOD cross-linked with a mixture of bovine serum albumen (BSA) and gultaraldehyde (GLA).The optimum conditions for potentiometric bi-layer biosensor include a polymerization time of 300 s for the inner layer at an applied current density of 0.25 mA cm^{-2}, a drying time of 30 min for the outer layer, pH 7, and 0.025 MTris–HCl. Sensitive amperometric measurements obtained from PPy–PNP–XOD–$Fe(CN)_6^{4-}$ biosensors were compared with those of potentiometric measurements obtained from PPy–NO_3/BSA/GLA–PNP–XOD bi-layer biosensor. A minimum detectable concentration of 20.0 μM phosphates and a linear concentration range of 20–200 μM were achieved with potentiometric PPy–NO_3/BSA/GLA–PNP–XOD 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 PPy–PNP–XOD–$Fe(CN)_6^{4-}$ amperometric and PPy–NO_3/BSA/GLA–PNP–XOD potentiometric bi-biosensors, therefore, they will not have any effect on phosphate measurement in both biosensors at levels normally present in water. PPy–NO_3/BSA–GLA–PNP–XOD potentiometric biosensor was used to analyse phosphate in real samples.

1. Introduction

Large quantities of phosphate have been used for detergents and for the treatment of boiler waters to prevent scale formation, therefore, it has become necessary to determine micro/trace amounts of phosphates in waters, such as drinking waters, natural waters, waste waters and polluted waters that are discharged from various sources. High phosphate concentration can pollute water resources and causes eutrophication of lakes and rivers (Nakamura et al., 2010). The eutrophication of water by phosphate can lead to over-growth of plants and toxic algae, thereby making it unsuitable for drinking or industrial use and also making water ways inaccessible (Keup, 1968). In semiconductor industries, phosphorus existing at trace/ultratrace amounts in the water can damage the quality of the semiconductors, and therefore the amounts must be lowered as much as possible. There has been a growing demand for highly sensitive, accurate and rapid determination, as well as simple and onsite analysis of phosphate (Motomisu and Li, 2005).

Phosphate determination is also important in clinical diagnosis; the determination of phosphate in body fluid provides useful informat, ion about certain diseases and about the energetic state of cells and bone function (Kivlehan et al., 2009; Shervedani and Pourbeyram, 2009).

Optical instrumental methods are sensitive and mainly used for the laboratory determination of phosphate and they have detection limit between 20 and 150 nM. Molybdate/rodamine fluorescence method gave a detection limit of 20 nM, but they are laborious and prone to interference, unstable or erratic measurement and lack of selectivity due to the fact that some reagents can produce emission for more than one analyte.

Phosphate determination based on spectroscopy (Galhardo and Masini, 2000; Mckelvie, 2000; Li et al., 2002; Nakamura et al., 2004; Yaqoob et al., 2004; Lin et al., 2006; Okoh et al., 2006; Gimbert et al., 2007; Nevesa et al., 2008; Yaqoob et al., 2008) and chromatographic techniques (Galceran et al., 1993; Bello and González, 1996;

* Corresponding author. Present address: Environmental and Industrial Chemistry Unit, Department of Chemical Sciences, Fountainn University, Osogbo, Nigeria. Tel.: +61 242742664.
E-mail address: Abdulazeez.Lawal@det.nsw.edu (A.T. Lawal).

0956-5663/$ - see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bios.2012.08.012

Colina et al., 1996; Zhu et al., 2008) were the analytical techniques commonly used for phosphate site monitoring. 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) and cobalt metallic wires (Chen et al., 1997, 1998; De Marco and Phan, 2003; Gimbert et al., 2007; Bai et al., 2011). Other methods used for determination of phosphate included flow injection systems based on luminescence (Kyung et al., 2009; Andolina and Morrow, 2010; Cardemil et al., 2011), chemiluminescence (Kawasaki et al., 1989; Nakamura et al., 1999a,b, 2003; Yaqoob et al., 2004; Motomisu and Li, 2005), florescence reactions (Motomisu and Li, 2005; Wang et al., 2010; Kim et al., 2011), conductometry (Zhang et al., 2008) and screen printed electrodes (Kwan et al., 2005; Tanimoto de Albuquerque and Ferreira, 2007; Zou et al., 2007; Khaled et al., 2008).

An alternative to time-consuming and laborious phosphate ion determination using classical methods (Colina et al., 1996; Bello and Gonzallez, 1999; Mckelvie, 2000) is the use of a conducting polymer biosensor, which is able to measure the substrate directly in the sample (D'Urso and Coulet, 1993; Cosnier and Gondran, 1998; Roger et al., 2003, 2005; Adeloju and Lawal, 2005, 2011; Rahman et al., 2006; Akyilmaz and Yorganci, 2007; Barsan and Bratt, 2008; Lawal and Adeloju, 2012). With the advent of enzyme-based biosensors, several approaches have been investigated for detecting phosphate ion (Haemmerli et al., 1990; Conrath et al., 1995; Menzel et al., 1995; Roger et al., 2003; Akyilmaz and Yorganci, 2007; Adeloju and Lawal, 2011). 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 et al., 1990; Male and Luong, 1991; Wollenberger et al., 1992; D'Urso and Coulet, 1993; Su and Mascini, 1995; Chen et al., 1997; Tzanavaras and Themelis, 2002; Vazquez et al., 2003) and most commonly used alkaline phosphatase. Others are acid phosphatase (Guilbault and Nanjo, 1975; Guilbault, 1984), glucose oxidase (Su and Mascini, 1995; Zhiqiang et al., 2008), pyruvate oxidase (Mori et al., 1994; Ikebukuro et al., 1996; Mak et al., 2003; Roger et al., 2005; Rahman et al., 2006; Akyilmaz and Yorganci, 2007), sucrose phosphorylase, phosphoglucomutase and glucose 6-phosphate dehydrogenase. Combination of maltose phosphorylase (MP), mutarotase (MR) and glucose oxidase (GO*x*) have been used for fabrication of phosphate biosensors (Mousty et al., 2001; Zhiqiang et al., 2008).

Various enzyme immobilization methods, such as adsorption, covalent bonding, entrapment and cross-linking, have been used to immobilize the above enzymes. Of these, the use of cross-linking is favored by many researchers due to the simplicity it offers for direct immobilization of relevant phosphate enzymes onto different electrodes (Guilbault, 1984; Watanabe et al., 1987; Watanabe et al., 1988; D'Urso et al., 1990; D'Urso and Coulet, 1993; Konishita et al., 1995). Some specific examples of phosphate biosensors fabricated by cross-linking of enzymes include the use of gultaraldehyde (GLA) with or without bovine serum albumin (BSA) to immobilize xanthine oxidase (XOD) and purine nucleoside phosphorylase (PNP) on nylon, teflon membrane and cellulose acetate membrane (Watanabe et al., 1988; Konishita et al., 1995). Lawal and Adeloju reported a study on the use of this chemical cross-linking method with polypyrrole (PPy) films in a bi-layer arrangement for the development of a phosphate biosensor and they successfully immobilized XOD and PNP into polypyrrole films (Lawal and Adeloju, 2009a, 2009b, 2010).

Hybrid designs with two or more polymer layers (i.e. PPy or overoxidized PPy and BSA–GLA) offer a remarkable solution for interferant rejection (Patano and Kuhr, 1995; Adeloju and Lawal, 2011). When used with a BSA–GLA matrix, the permselective qualities of a multilayer configuration are coupled with the high enzyme loading and long-term stability of cross-linking with BSA and GLA (Guerrieri et al., 1998). Unlike, monolayer arrangements (Bartlett et al., 1992; Patano and Kuhr, 1995; Guerrieri et al., 1998), a hybrid arrangement can improve the long-term stability and improve the sensitivity and selectivity of the biosensor.

An 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 10 years as a result of its high selectivity and the sensitivity of amperometric signal (Roger et al., 2003; Tanimoto de Albuquerque and Ferreira, 2007; Lawal and Adeloju, 2009b). 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, 2011; Lawal and Adeloju, 2009b). 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 is avoided Lawal and Adeloju (2012) and Villalba et al. (2009) recently reviewed the advantages and disadvantages of the electrochemical biosensors for the determination of phosphate.

The PNP–XOD bienzyme system employed recently in various studies (Cosnier and Gondran, 1998; Adeloju and Lawal, 2005, 2011; Lawal and Adeloju, 2009a, 2009b) showed that 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 et al. (1992) employed amplification by enzymatic substrate recycling in order to lower the detection limit, involving co-immobilization 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 recognized by sequential oxidation by XOD.

Hypoxanthine was subsequently oxidized to H_2O_2, catalysed by XOD (Watanabe et al., 1987; D'Urso et al., 1990; Wollenberger et al., 1992; D'Urso and Coulet, 1993), as given

$$\text{Inosine} + \text{orthophosphate} \xrightarrow{\text{PNP}} \text{ribose}-1-\text{phosphate} + \text{hypoxanthine} \quad (1)$$

and

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

In this study, biosensors were developed based on the enzymatic reaction shown in Eqs. (1) and (2). We also compare the fabrication of a novel potentiometric bi-layer biosensor for phosphate by the use of a composite bi-layer arrangement, as PPy–NO_3/BSA–GLA–PNP–XOD, consisting of an inner electropolymerized PPy–NO_3 layer and an outer layer of PNP and XOD cross-linked with a mixture of BSA and GLA with PPy–PNP–XOD–$Fe(CN)_6^{4-}$ amperometric biosensor. The PPy–NO_3/BSA–GLA–PNP–XOD biosensor combines the advantages of cross-link immobilization, such as high enzyme loading and long-term stability of the enzymes, with the excellent interferant rejection of electrosynthesized polypyrrole film. Important considerations in the development of the PPy–NO_3/BSA–GLA–PNP–XOD biosensor include influence of drying time, PNP: XOD

ratio, GLA and BSA concentrations. In PPy–NO_3/BSA–GLA–PNP–XOD biosensor, we investigated the effect of enzyme (PNP: XOD) mole ratio, pH, inosine and buffer concentrations, analytical characteristic, stability and common interferences affecting the two electrochemical biosensors.

2. Materials and methods

2.1. Reagents and standard solutions

XOD (EC.1.2.3.2.2 Grade1 (2.0 U mg^{-1})) from buttermilk, PNP (EC.2.4.2.1.2 (15 U mg^{-1})), potassium ferrocyanide, and pyrrole were obtained from Sigma Sydney. Pyrrole was distilled under vacuum at 130 °C prior to use, and this was stored in an aluminum 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 analytical grade. A 0.1 M sodium chloride solution was prepared by dissolving an appropriate amount (1.5 g) of NaCl in Milli-Q water. The volume was then adjusted to 250 mL. A stock solution of 0.25 M $K_4Fe(CN)_6$ salt was prepared by dissolving 1.0060 g of the salt in Milli-Q water prepared without further purification. Enzyme stock solutions were stored in the fridge and freezer, respectively, until required. Phosphate stock solution (0.5 M) was stored in the refrigerator and was diluted when necessary to give the required standard concentration.

2.2. Instrumentation

A potentiostat/galvanostat designed and constructed in our laboratories was used for electrochemical measurements. The potentiostat/gavanostat was used in galvanostatic mode for the electropolymerization. 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.

2.3. Enzyme immobilization

2.3.1. Electrode preparation

A 320 μm aluminum 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 aluminum 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 aluminum oxide. The electrode was dried with fiber-free tissue paper and fixed onto a retort stand for the next step.

2.3.2. Electropolymerization of PPy–PNP–XOD–$Fe(CN)_6^{4-}$ 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 electropolymerization of PPy film. PPy–PNP–XOD–$Fe(CN)_6^{4-}$ biosensor was made by immobilization of xanthine oxidase (6.2 U/mL), purine nucleoside phosphorylase (48 unit/mL) and 50 mM potassium ferrocyanide ($K_4Fe(CN)_6$) into a polypyrrole film. The electropolymerization 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 polymerization time of 200 s. The polymer electrode formed after galvanostatic polymerization 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.

2.3.3. Polypyrrole–nitrate with ferrocyanide film

The galvanostatic electropolymerization of the PPy–NO_3–$Fe(CN)_6^{4-}$ film inner layer was fabricated using a three-electrode voltammetric cell. The working electrode was a platinum electrode (0.03 cm^2), while a platinum wire and an Ag/AgCl were used as the auxiliary and reference electrodes, respectively. Pyrrole concentration was varied and the optimum concentration was used for inner layer. The PPy–NO_3 film was formed in a monomer solution containing 0.4 M pyrrole, 20 mM PPy–NO_3–$Fe(CN)_6^{4-}$and 0.1 M KNO_{l3} with an applied current density of 0.25 mA cm^{-2} for various polymerization times. After the galvanostatic film formation, the polymer electrode was washed several times under a stream of Milli-Q water to remove remaining monomer solution. The formation of the above films was followed by the formation of an outer layer by the immobilization of PNP and XOD with a BSA and GLA mixture. Three microlitres of this mixture which contained all the above components was spread onto the inner PPy layer. This top layer was allowed to air dry until the film mixture had gelatinized and hardened. The electrode was washed under a stream of Milli-Q water to remove any loosely bound molecules, prior to use for analysis.

2.4. 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.

2.5. Potentiometric measurements

The electrode was rinsed thoroughly with distilled water to remove any loosely bound enzymes after immobilization in BSA/GLA. 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.

2.6. Phosphate analysis in water samples

The bi-layer biosensor and reference electrodes were immersed in 25 mL of buffer solution (0.05 M of Tris–HCl, pH 7 containing 0.1 M KCl and 5 mM inosine) with constant stirring. After a constant potential was obtained (equilibration time: 24 min) 100 mL of raw water sample was added. When a steady-state potential was reached 100 mL of first phosphate standard solution was added. Then second and third standard solutions were added sequentially, allowing for the electrode potential to stabilize before the next addition. The phosphate concentration in the water sample was calculated by the standard addition method.

3. Results and discussion

3.1. Response to phosphate

In amperometric biosensor, XOD in the presence of molecular oxygen oxidized H*x* 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 or the sensitivity of the electroactive polymer layer to change in the pH of the solution. Fig. 1 shows a typical potentiometric response of the bi-layer potentiometric biosensor upon the successive addition of phosphate into electrochemical cell with stirring. As expected, the potentiometric response decreased with increasing phosphate concentration due to the increasing generation of hydrogen peroxide. It has been shown in a previous study (Adeloju and Moline, 2001) that the potential of another polypyrrole biosensor decreases with increasing the concentration of hydrogen peroxide and solution pH.

3.2. Influence of pyrrole concentration on PPy film formation in inner layer

Fig. 2 shows that the increasing pyrrole concentration used for polymerization of the inner PPy layer resulted in an increase in the potentiometric response for phosphate, reaching an optimum sensitivity in the presence of 0.4 M pyrrole. No further increase in sensitivity was observed beyond this pyrrole concentration, possibly due to the formation of thicker polypyrrole film which will increase the diffusion barrier.

3.3. Effect of PNP and XOD ratio

The performance of biosensors for environmental monitoring in terms of detection limit, stability and calibration range was strictly dependent on the enzyme loading. Both biosensors show that the optimum response was obtained when a mole ratio of 1:8

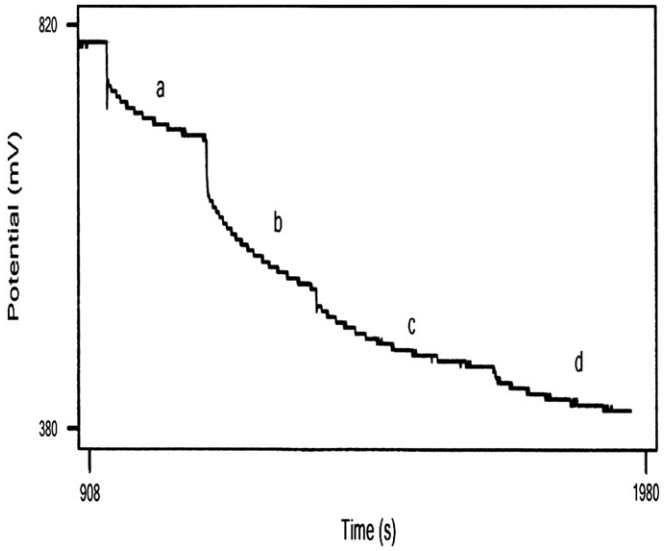

Fig. 1. Typical responses obtained for the quantification of phosphate in Gippsland Lake with PPy–NO_3/$Fe(CN)_6^{4-}$/BSA–GLA–PNP–XOD biosensor. Phosphate concentration (a) sample only, (b) +0.09, (c) +0.20 and (d) +0.29 mM. Potentiometric measurement was made in 0.05 M Tris–HCl buffer, and [Phosphate] was 10 mM.

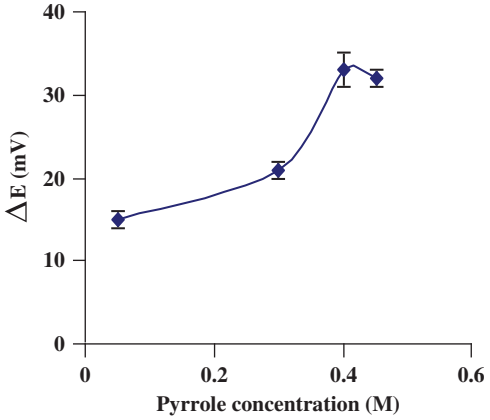

Fig. 2. Effect of PPy concentration used for the formation of film in layer 1 on the potentiometric response of phosphate obtained with bi-layer PPy–NO_3/$Fe(CN)_6^{4-}$/BSA–GLA–PNP–XOD. Potentiometric measurement was made in 0.05 M Tris–HCl buffer, and [Phosphate] was 10 mM.

of XOD: PNP was incorporated into PPy and the outer layer of BSA/GLA. This is very similar to the mole ratio reported by D'Urso and Coulet (D'Urso et al., 1990; D'Urso and Coulet, 1993) for a phosphate biosensor. Guilbault (Guilbault and Cserfalvi, 1976) reported a ratio of 1:10, while Kulys (Kulys et al., 1992) and Wollenberger (Wollenberger et al., 1992) reported a ratio of 1:5 for their construction of an amperometric phosphate biosensor. On the other hand, Yao (Yao et al., 2003) reported an optimum ratio of 1:21, while Konishita et al. (1995) found a ratio of 1:3 to be the optimum ratio for their amperometric phosphate biosensor.

3.4. Effects of pH and buffer concentration

Fig. 3 shows the effect of pH on the response to phosphate by both amperometric and potentiometric PPy–PNP–XOD biosensors. The optimum pH of the sensors was found to be 8 close to the optima of the two enzymes in solution (Berger et al., 1989). Guilbault et al. (Guilbault and Nanjo, 1975) and Kulys et al. (1992) found that pH 7 produced the optimum sensitivity for an amperometric phosphate biosensor. While D'Urso and Coutlet (D'Urso et al., 1990) and Yao (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 immobilization, or if the sample did not contain inosine, which had to be supplied in excess in a phosphate-free buffer. Wollenberger et al. (1992) used excess inosine to ensure a co-reactant independent of phosphate response was obtained. The influence of the concentration of buffer solution on the biosensor's response is shown in Fig. 4. In amperomeric 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.

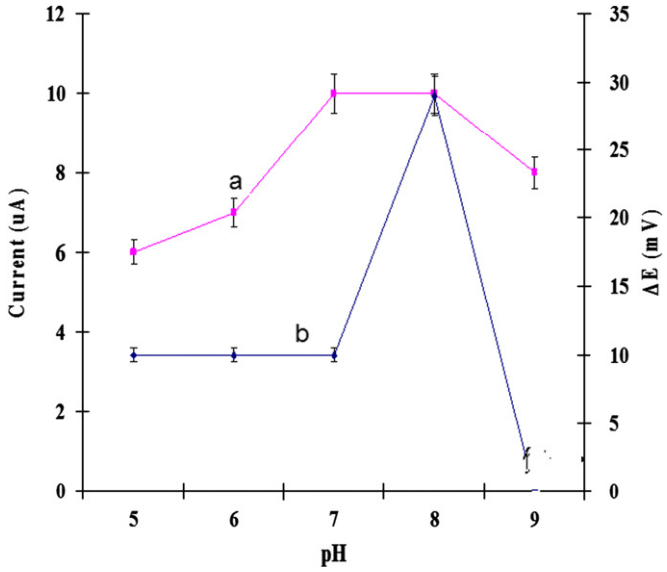

Fig. 3. Effect of pH on phosphate response obtained with both amperometric PPy–PNP–XOD–Fe(CN)$_6^{4-}$ (a) and potentiometric PPy–NO_3/Fe(CN)$_6^{4-}$/BSA–GLA–PNP–XOD and (b) $^{-}$ biosensors. [Phosphate] was 10 mM. The monomer solution contained 0.4 M pyrrole, 6.2 U/mL of XOD, current density: 0.5 mA/cm^2 polymerization period: 200 s, and 50 mM Fe(CN)$_6^{4-}$ drying time 30 min.

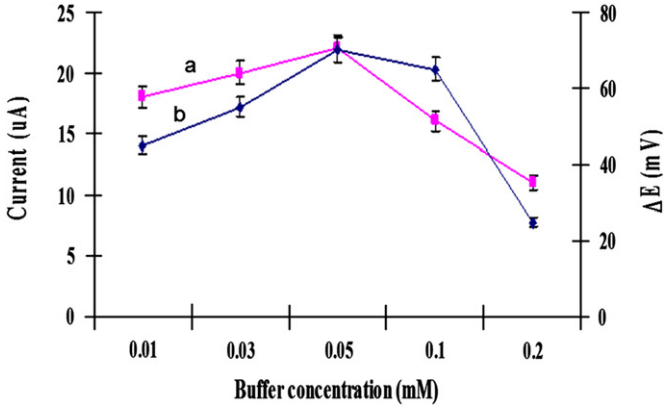

Fig. 4. Effect of buffer concentration on phosphate response obtained with both amperometric PPy–PNP–XOD–Fe (CN)$_6^{4-}$ (a) and potentiometric PPy–NO_3/Fe(CN)$_6^{4-}$/BSA–GLA–PNP–XOD and (b) $^{-}$ biosensors. [Phosphate] was 10 mM. The monomer solution contained 0.4 M pyrrole, 6.2 U/mL of XOD, current density: 0.5 mA/cm^2 polymerization period: 200 s, and 50 mM Fe(CN)$_6^{4-}$ drying time 30 min.

3.5. Effect of drying time

The drying of the outer layer is critical for obtaining reliable measurement with the bi-layer PPy–NO_3/BSA–GLA–PNP–XOD electrode. Fig. 5 shows that the phosphate response obtained with this electrode increased with the drying time up to 30 min and beyond this drying time, the response declined slightly and remained constant. This may be due to changes in the permeability of the outer layer with increased drying time.

3.6. Analytical characteristics and stability

Fig. 6 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 0.1 mM. On the other hand, the calibration curve obtained for bi-layer potentiometric biosensor is as

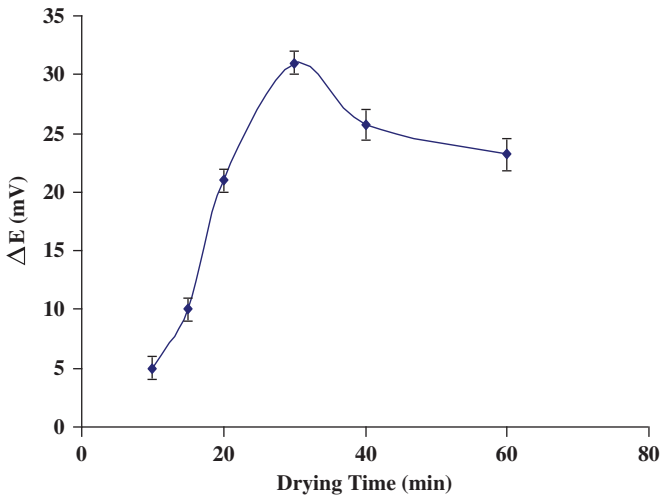

Fig. 5. Effect of drying of layer 2 on the potentiometric response of phosphate obtained with bi-layer PPy–NO_3/Fe(CN)$_6^{4-}$/BSA–GLA–PNP–XOD. [Phosphate] was 10 mM.

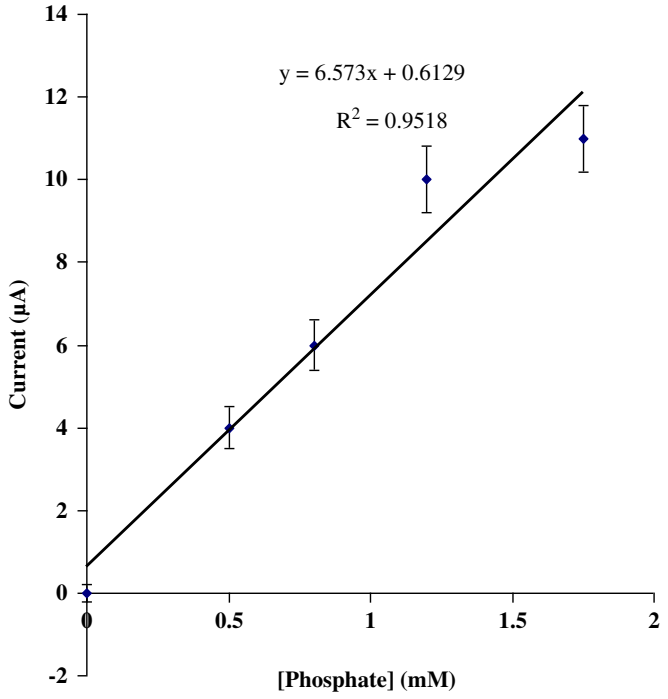

Fig. 6. Typical calibration graph obtained for phosphate with amperometric PPy–PNP–XOD–Fe(CN)$_6^{4-}$ biosensor. [Phosphate] was 10 mM. The monomer solution contained 0.4 M pyrrole, 6.2 U/mL of XOD, current density: 0.5 mA/cm^2 polymerization period: 200 s and 50 mM Fe(CN)$_6^{4}$.

shown in Fig. 7. The bi-layer PPy–NO_3/BSA–GLA–XOD–PNP biosensor is very sensitive, enabling the achievement of a minimum detectable amount of 20 μM and a linear concentration range of 20–200 μM for phosphate. These detectable amount are better than those reported previously (Watanabe et al., 1988; Haemmerli et al., 1990; Kulys et al., 1992) with biosensors based on the use of the same bienzyme system. These limits of detection are higher 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 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). The bi-layer arrangement of PPy–NO_3/BSA–GLA–XOD–PNP biosensor appeared to be useful in minimizing interference and

increasing enzyme loading beyond the amount that can be achieved in a single layer amperometric (PNP–XOD–$Fe(CN)_6^{4-}$ biosensor. These limits are enough to probe phosphate concentration within inland water ways. Table 1 shows analytical characteristic of some conventional methods of phosphate sensing.

Bi-layer PPy–NO_3/BSA–GLA–XOD–PNP biosensor was found to be stable for 25 day, achieving a reproducibility of 4.8% rsd ($n=5$) for potentiometric measurements during this period. However, the potentiometric response of the bi-layer biosensor decreased by 10% after 25 day, possibly due to the gradual leaching of enzyme into buffer solution or deactivation of the enzymes. Nevertheless, the stability of the PPy–NO_3/BSA–GLA–XOD–PNP biosensor is still much better than those of other phosphate biosensors reported by Menzel et al. (1995). Male and Luong (1991) reported that the use of a bienzyme system on reactivated nylon membrane resulted in a loss of 30% of its response in 3 weeks. The stability of the PPy–NO_3/BSA–GLA–XOD–PNP bi-layer biosensor is far superior to that achieved with the single layer amperometric (PNP–XOD–$Fe(CN)_6^{4-}$ biosensor which lost 50% of its initial sensitivity after only 8 day and stabilized at 20% of its initial sensitivity after 2 weeks. However, when the PPy–NO_3/BSA–GLA/BSA–GLA–XOD–PNP bi-layer biosensor is stored in a buffer solution, the enzyme stability is improved and optimum potentiometric response is obtained.

3.7. Interference study and analysis of phosphate in water samples

Fig. 8 shows the effect of ascorbic acid and uric acid on phosphate response obtained with PPy–NO_3/BSA–GLA/BSA–GLA–XOD–PNP bi-layer biosensor. The addition of uric acid, ascorbic acid and glycine at 0–5 mM did not have any significant interference effect on the response on both biosensors. Ascorbic acid concentration greater than 2.5 mM enhanced the response by up to 4.0%, while uric acid suppressed the signal by 3.6% compared to single layer 42% enhancement and 17% depression (Su and Mascini, 1995; Lawal and Adeloju, 2010). Evidently the presence of the inner layer PPy–NO3 layer reduced the interference of both ascorbic and uric acids on the phosphate response. The observed enhancement and depression are due to the penetration of ascorbic and uric acids through the PPy film to the electrode surface and subsequent increase or decrease in potentiometric signals.

The linear range (20–200 μM) of PPy–NO_3/$Fe(CN)_6^{4-}$/BSA–GLA–PNP–XOD biosensor is wider than those obtained in our laboratories with single layer PPy–XOD–PNP and BSA–GLA–XOD–PNP biosensors. Evidently, the use of bi-layer arrangement helps to extend the analytical applicability of the potentiometric biosensor for phosphate measurement. The results in Table 2

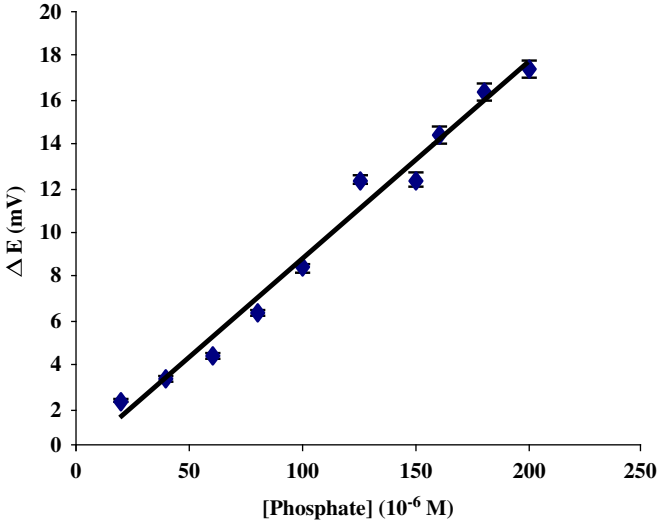

Fig. 7. Calibration curve for phosphate on the PPy-NO_3/$Fe(CN)_6^{4-}$/BSA–GLA–PNP–XOD electrode.

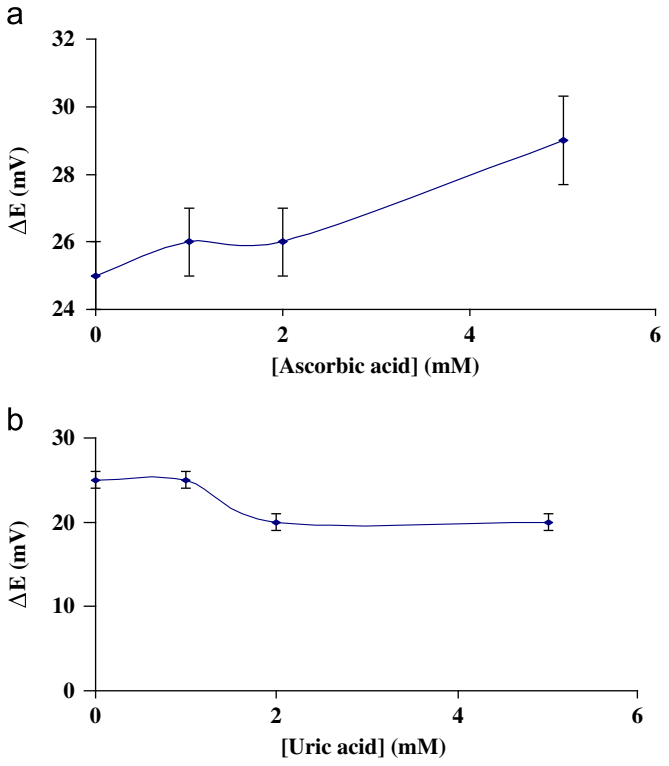

Fig. 8. Effect of (a) ascorbic acid on the potentiometric response of phosphate. (b) Effect of uric acid on the potentiometric response of phosphate obtained with the bi-layer PPy–NO_3/$Fe(CN)_6^{4-}$/BSA–GLA–PNP–XOD biosensor. [Phosphate] was 10 mM.

Table 1
Some conventional methods for determination of inorganic and organic phosphate.

Methodology	Reagent	LOD (μm)	Reference
ISE	Mplybdate complex	0.6	Ganjali et al. (2006)
Ion chromatography		0.1	Anthony et al. (2002)
Amperometric	Molybdate complex	3	Abdulla et al. (1989)
Amperometric	NPN, XOD, AP	2	Abdallah et al. (1991)
Fluorescent probe	PVC matrice	5	Lin et al. (2006)
Florescent PVC matrix	NP, XOD, HRP Almorine	3	Vazquez et al. (2003)
Potentiometric	NPN/XOD	20	Lawal and Adeloju (2010, 2012)
Fluorescent	Molybdate and thamine		Zang and Beck (2001)
Amperometric	MP, MR, GODx, AP	0.01	Mousty et al. (2001)
Capacitance	Malachite green	0.2	O'Toole et al. (2007), Basheer et al. (2011)
Conductometry	AP/BSA/GLA	0.4	(Guedri and Durrieu 2009)

Table 2
Determination of phosphate in water sample with the bi-layer PPy–NO_3/$Fe(CN)_6^{4-}$/BSA–GLA–PNP–XOD biosensor.

Location	Phosphate concentration (mg/L)
Warragamba 47	4.70 ± 0.42
Warragamba 48	6.32 ± 0.63
Lake Illawarra Warrawong Yacht Club	3.17 ± 0.92
Lake Illawarra Berkeley Fish Market	3.90 ± 0.50
UWS Kingswood Swamp	5.92 ± 0.40
Werrington Swamp	6.32 ± 0.91
Gippsland Lakes	
17M	7.11 ± 1.20
14M	1.58 ± 0.08
15M	2.77 ± 0.07
15F	1.58 ± 0.03
Recommended value ANZEC	0.046
WHO	1.00

Uncertainty margins ($\pm$) were calculated from mean deviation of replicates.

show the phosphate levels in all the water samples analyzed with the PPy–NO_3/$Fe(CN)_6^{4-}$/BSA–GLA–PNP–XOD, these results demonstrate that accurate and sensitive determination of phosphate can be accomplished with the biosensors in water samples that are free of interferants. The standard deviation ($n=3$) values also demonstrate that the biosensor enables reproducible quantitative phosphate analysis. Bi-layer potentiometric PPy–NO_3/BSA–GLA–XOD–PNP biosensors compares favorably well with the other methods used in quantitative analysis of similar water samples. Nakamura et al. (1999a,b) obtained 9.5 mg/L when River Tone was analysed using chemilumenesence method which is close to 7.8 mg/L obtained for Gipsland lakes (17F) using bi-layer biosensor. McGraw et al. (2007) obtained 1.5 mg/L when Dublin river was analysed with automated instrument which is also similar to some results obtained for Gippsland lake (samples 14 F and 15 M). Nakamura et al. (2003)'s result of phosphate analysis of river Watarase (1.45–2.48 mg/L) using chemilumenescence are closed to the result obtained when Gippsland lakes (1.58–2.77 mg/L) were analysed using bi-layer phosphate biosensor.

4. Conclusions

Amperometric PPy–PNP–XOD–$Fe(CN)_6^{4-}$ and bi-layer potentiometric PPy–NO_3/BSA–GLA–XOD–PNP biosensors have been fabricated for accurate amperometric and potentiometric measurements of phosphate at concentrations that are suitable for environmental monitoring. Potentiometric biosensor can detect a minimum of 20 μM phosphate ion and has a linear concentration range of 20–200 μM, whereas amperometric biosensor can detect a minimum of 0.1 mM 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 bi-layer arrangement appeared to be useful in minimizing interference and increasing enzyme loading beyond the amount that can be achieved in a single layer amperometric (PNP–XOD–$Fe(CN)_6^{4-}$ biosensor.

References

Abdallah, A.M., Khalifa, M.E., et al., 1991. Analytica Chimica Acta 251, 207.
Abdulla, H.M., Greenway, G.M., et al., 1989. Analyst 114, 1575.
Adeloju, S.B., Lawal, A., 2005. International Journal of Environmental Analytical Chemistry 85 (9–11), 771–780.
Adeloju, S.B., Lawal, A.T., 2011. Analytica Chimica Acta 691 (1–2), 89–94.
Adeloju, S.B., Moline, A.N., 2001. Biosensors and Bioelectronics 16, 133–139.
Akyilmaz, E., Yorganci, E., 2007. Electrochimica Acta 52 (28), 7972–7977.
Andolina, C.M., Morrow, J.R., 2010. European Journal of Inorganic Chemistry 1, 154–164.
Anthony, P.J., Karthikeyan, S., et al., 2002. Journal of Chromatography B 767, 363–368.
Bai, Y., Tong, J., et al., 2011. Xi'an 483, 559–564.
Barsan, M.M., B. C. M. A, 2008. Talanta 74 (5), 1505–1510.
Bartlett, P.N, A. B., Eastwickfield, E.V.J., 1992. Journal of the Chemical Society, Faraday Transactions 88, 2677.
Basheer, S., Samyn, D., et al., 2011. Biosensors and Bioelectronics 27 (1), 58–63.
Bello, M., Gonzallez, A.G., 1999. Analysis 27, 97.
Bello, M, González, N., 1996. Journal of Chemistry 73, 1174–1176.
Berger, H.U., Grass, I.M., et al., 1989. Method of Enzymic Analysis. Wiley-VCH, Weinheim.
Cardemil, C.V., Smulski, D.R., et al., 2011. DNA and Cell Biology 29 (9), 519–531.
Chaniotakis, N., Jurkschat, K, et al., 1993. Analytica Chimica Acta 282, 345–352.
Chen, Z., De Marco, R., et al., 1997. Analytical Communications 34, 93.
Chen, Z., Grierson, P., et al., 1998. Analytica Chimica Acta 363 (2–3), 191–197.
Colina, M., Ledo, H., et al., 1996. Journal of Chromatography A739, 223.
Conrath, N., Gründig, B., et al., 1995. Analytica Chimica Acta 309, 47–52.
Cosnier, S., Gondran, C., 1998. Analytical Chemistry 70, 3952–3956.
D'Urso, E., Coulet, P., et al., 1990. Analytica Chimica Acta 239, 1–5.
D'Urso, E.M., Coulet, P.R., 1993. Analytica Chimica Acta 281 (3), 535–542.
De Marco, R., Phan, C., 2003. Talanta 60, 1215–1221.
Galceran, M.T., Diez, M., et al., 1993. Journal of Chromatography A 657 (1), 77–85.
Galhardo, C.X., Masini, J.C., 2000. Analytica Chimica Acta 417 (2), 191–200.
Ganjali, M.R., Norouzi, P., et al., 2006. Analytica Chimica Acta 567 (2), 196–201.
Gimbert, L.J., Haygrath, P.M., et al., 2007. Talanta 71, 1624–1628.
Guedri, H., Durrieu, C., 2009. Sensor Letters 7 (5), 788–794.
Guerrieri, A., De BeneDetto, G.E., et al., 1998. Biosensors and Bioelectronics 13, 103.
Guilbault, G., 1984. Analytical Uses of Immobilised Enzyme. Dekker, New York.
Guilbault, G., Cserfalvi, T., 1976. Ion selective electrodes. Analytical Letters 9, 277–280.
Guilbault, G., Nanjo, M., 1975. Analytica Chimica Acta 78, 69–74.
Haemmerli, S.D., Suleiman, A.A., et al., 1990. Analytical Biochemistry 191 (1), 106–109.
Huwel, S., Haalck, L., et al., 1997. Enzyme and Microbial Technology 21 (6), 413–420.
Ikebukuro, A.K., Nishida, R., et al., 1996. Journal of Biotechnology 48 (1–2), 67–72.
Kawasaki, H., Sato, K., et al., 1989. Analytical Biochemistry 182 (2), 366–370.
Keup, L.L., 1968. Water Research 2, 373–386.
Khaled, E., Hassan, H.N.A., et al., 2008. Talanta 77 (2), 737–743.
Kim, K.L, Oh, D.J, et al., 2011. Chemistry—An Asian Journal 6 (1), 122–127.
Kivlehan, F., Mace, W.J., et al., 2009. Electrochimica Acta 54 (7), 1919–1924.
Konishita, H., Yoshida, D., et al., 1995. Analytica Chimica Acta 303, 301–303.
Kulys, J., Higgins, I.J., et al., 1992. Biosensors and Bioelectronics 7 (3), 187–191.
Kwan, R.C.H., Leung, H.F., et al., 2005. Analytical Biochemistry 343 (2), 3–267.
Kyung, K.I.M.S., Hoon, L.E.E.Dong, et al., 2009. Accounts of Chemical Research 42 (1), 23–31.
Lawal, A.T., Adeloju, S.B., 2009a. Biosensors and Bioelectronics 25 (2), 406–410.
Lawal, A.T., Adeloju, S.B., 2009b. Journal of Applied Science 9 (10), 1907–1914.
Lawal, A.T., Adeloju, S.B., 2010. Journal of Molecular Catalysis B: Enzymatic 63 (1–2), 45–49.
Lawal, A.T., Adeloju, S.B., 2012. Journal of Applied Science 20 (4), 315.
Li, y, Muo, y., et al., 2002. Analytica Chimica Acta 455 (2), 315–325.
Lin, X., Wu, X., et al., 2006. Talanta 70 (1), 32–36.
McGraw, C, Stitzel, S., Cleary, J., Slater, C., Diamond, D., 2007. Talanta 71, 1180–1185.
Mak, W.C., Chan, C., et al., 2003. Biosensors and Bioelectronics 19 (3), 233–237.
Male, K.B., Luong, J., 1991. Biosensors and Bioelectronics 6, 581.
Mckelvie, I.D., 2000. Handbook of Water Analysis. Makcel Dekker, New York.
Menzel, C., Lerch, T., et al., 1995. Analytica Chimica Acta 317 (1–3), 259–264.
Mori, H., Kogure, M., et al., 1994. Analytical Letters 27 (2), 309–321.
Motomisu, S., Li, Z.H., 2005. Talanta 66, 332.
Mousty, C., Cosnier, S., et al., 2001. Analytica Chimica Acta 443 (1), 1–8.
Nakamura, H., 2010. Analytical Methods 2 (5), 430–444.
Nakamura, H., H.M., et al., 2003. Analytical Letters 36 (9), 1805–1817.
Nakamura, H., Y.R., et al., 2004. Analytica Chimica Acta 518, 45–49.
Nakamura, H., Tanaka, H., et al., 1999a. Talanta 50 (4), 799–807.
Nakamura, H, Hasegawa, M, Nomura, Y, Ikeburuka, M, Karube, I, 1999b. Journal of Biotechnology 75, 127–133.
Nevesa, M.S.A.C., Soutob, M.R.S., et al., 2008. Talanta 77 (2), 527–532.
O'Toole, M., Lau, K.T., et al., 2007. Analytica Chimica Acta 597 (2), 290–294.
Okoh, M.P., Hunter, J.L., et al., 2006. Biochemistry 45, 14764.
Patano, P, Kuhr, W.G.E.C., 1995. Journal of Electroanalytical Chemistry 224, 405.
Petrucelli, G., Kawachi, E., et al., 1996. Analytical Communications 33, 227–229.
Rahman, M.A., Park, D.-S., et al., 2006. Biosensors and Bioelectronics 21 (7), 1116–1124.
Roger, C.H., Kwan, H.F, et al., 2005. Applied Microbiology and Biotechnology 66 (4), 377–383.
Roger, C.H., Kwana, H.F.L., et al., 2003. Biosensors and Bioelectronics 19 (3), 233–237.
Sasaki, S., Ozawa, S., et al., 2004. Talanta 63 (1), 131–134.
Shervedani, R.K., Pourbeyram, S., 2009. Biosensors and Bioelectronics 24 (7), 2199–2204.
Su, Y., Mascini, M., 1995. Analytical Letters 28, 1359–1378.
Tanimoto de Albuquerque, Y.D., Ferreira, L.F., 2007. Analytica Chimica Acta 596 (2), 210–221.

Tzanavaras, P.D., Themelis, D.G., 2002. Analytica Chimica Acta 467 (1–2), 83–89.
Vazquez, M.J., Rodriguez, B., et al., 2003. Analytical Biochemistry 320 (2), 292–298.
Villalba, M.M., McKeegan, K.J., et al., 2009. Journal of Molecular Catalysis B: Enzymatic 59 (1-3), 1–8.
Villamil-Ramos, R., Yatsimirsky, A.K., 2011. Chemistry Communications 47 (9), 2694–2696.
Wang, D., Zhang, X., et al., 2010. Organic and Biomolecular Chemistry 8 (13), 2923–2925.
Watanabe, E., Endo, H., et al., 1987. Biosensors 3 (5), 297–306.
Watanabe, E., Endo, H., et al., 1988. Biosensors 3, 581.
Wollenberger, U., Schuber, F., et al., 1992. Sensors and Actuators 7, 412–415.
Yao, T., Takashima, K., et al., 2003. Talanta 60 (4), 845–851.
Yaqoob, M., Nabi, A., et al., 2004. Analytica Chimica Acta 510 (2), 213–218.
Yaqoob, M., Nabi, A., et al., 2008. Analytica Chimica Acta 615 (1), 73–79.
Zang, H., Beck, H.P., 2001. Analytical Letters 34, 2721.
Zhang, Z.Q., Jaffrezic-Renault, N., et al., 2008. Analytica Chimica Acta 615 (1), 73–79.
Zhiqiang, Z., Jaffrezic-Renaulta, Nicole, et al., 2008. Analytica Chimica Acta 615 (1), 73–79.
Zhu, M., Xie, B., et al., 2008. Journal of Pharmaceutical and Biomedical Analysis 48 (5), 1417–1424.
Zou, Z., Han, J., et al., 2007. Biosensors and Bioelectronics 22 (9 10), 1902–1907.