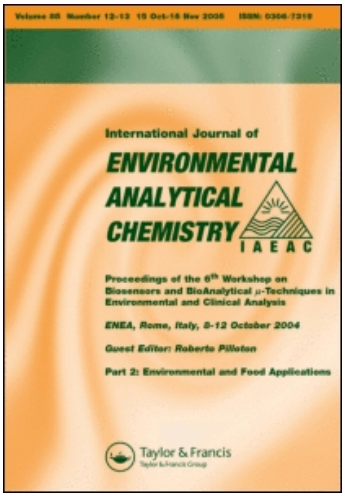

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Polypyrrole-based bilayer biosensor for potentiometric determination of phosphate in natural waters

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A method is described for the fabrication of a novel polypyrrole-based potentiometric bilayer biosensor for phosphate, 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. The optimum conditions for reliable utilization of the 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 M Tris–HCl. As little as 20 μM of phosphate was detected with the PPy-NO_3/BSA–GLA–PNP–XOD biosensor and the response was linear between 20 and 200 μM. Excellent recoveries (98.5–100.5%) of phosphate in lake, swamp and river water samples were obtained with the biosensor.

Keywords: Biosensor; Natural waters; Phosphate; Polypyrrole; Bilayer

1. Introduction

Various phosphate biosensors have been developed based on enzymatic sequences where a first enzyme (usually a phosphorylase) uses phosphate as a co-substrate to generate a product that is a substrate for a second enzyme (usually an oxidase). Among these are phosphate biosensors that use as biorecognition elements, substances such as nucleoside phosphorylase and xanthine oxidase [1–6], alkaline or acid [7, 8], phosphatase and glucose oxidase, pyruvate oxidase [9, 10] and sucrose phosphorylase [11], phosphoglucomutase [12] and glucose 6-phosphate dehydrogenase [11]. In particular, phosphate biosensors based on the use of a two enzyme system, which contained purine nucleoside phosphorylase (PNP) and xanthine oxidase (XOD) have gained considerable interest [2, 3, 5, 6, 13–17]. D'Urso and Coulet [2, 3] used the bienzyme system to improve the detection limit for phosphate by a 100-fold. In this case PNP catalyses the conversion of phosphate ions to hypoxanthine, as an intermediate

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product, while the second enzyme (XOD) catalyses the hyrolysis of hypoxanthine to hydrogen peroxide and uric acid:

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

and

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

Evidently, XOD plays the role of a biological amplifier, generating 3 mol of electroactive species (2 mol of peroxide and 1 mol of uric acid) for 1 mol of phosphate. This bienzyme system is usually immobilized on oxygen or platinum electrode and the oxygen consumed or H_2O_2 produced, as indicated by the above equations is detected amperometrically. Consumption of oxygen will lead to a reduction in current, while the production of peroxide will result in an increase in current. The decrease or increase in the measured current is often proportional to the phosphate concentration. In most cases, the peroxide produced is detected amperometrically and the magnitude of the response is related to the phosphate concentration.

The development of these phosphate biosensors has involved the use of various enzyme immobilization methods, such as adsorption, covalent bonding, entrapment and cross-linking. Of these, the use of cross-linking is favoured by many researchers due to the simplicity it offers for direct immobilization of relevant phosphate enzymes onto different electrodes [2, 3, 7, 13, 15]. 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 [13, 15]. However, to our knowledge there is no reported study on the use of this chemical cross-linking method with polypyrrole (PPy) films in a bilayer arrangement for the development of a phosphate biosensor. Also, to date, most of the reported phosphate biosensors are based on the amperometric measurement of the H_2O_2 liberated by the enzymatic reaction. Yet, if feasible, potentiometric mode of detection will simplify the design of such biosensor and the measurement process considerably, requiring only the use of two electrodes and only potential measurement.

In this article we report on the fabrication of a novel potentiometric biosensor for phosphate by use of a composite bilayer 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. This device 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 addition, the influence of ascorbic acid and uric acid on the sensitivity of the PPy-NO_3/BSA–GLA–PNP–XOD biosensor will be discussed. The successful application of the biosensor to the determination of phosphate in a range of natural water samples will also be demonstrated.

2. Experimental

2.1 *Reagents and standard solutions*

Xanthine oxidase (XOD) (EC.1.2.3.2.2 Grade 1) from buttermilk, purine nucleoside phosphorylase (PNP) (EC.2.4.2.1), inosine, potassium ferrocyanide and pyrrole were obtained from Sigma-Aldrich. All other chemicals were of analytical reagent grade, unless specified otherwise, and all compounds used in this work were prepared without further purification. The 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.

Barbitone buffer stock solution (0.5 M, pH 7.8) was prepared by neutralizing 0.5 M barbituric acid with 0.1 M sodium hydroxide. This was stored in the fridge and diluted when needed. A 0.1 M sodium chloride 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. The volume was then adjusted to 10 mL. The volumetric flask was then placed in an ultrasonic-bath until the remaining crystal of the salt dissolved and the solution was clear. $K_4Fe(CN)_6$ also undergoes UV degradation. The solution was stored until required. Stock solution of XOD was prepared by adding 100 μL of Milli-Q water to 100 unit XOD bottle, while stock solution of PNP was also prepared by adding 200 μL of Milli-Q water to 200 unit PNP bottle. These 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*

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 electropolymerization of pyrrole as well as for the potentiometric and amperometric measurements. Potentiometric measurements were performed in a two-electrode cell. The potentiostat was connected to a computer controller (AMD-K6-400 mHz Celeron processor, 32 MB RAM, 8 gigabyte HD, Hansonl Monitor and Windows '98 software keyboard and mouse) and a brother HL-12707 network laser printer. Solution was stirred when necessary with a Sybron Thermolyne (model S-17410).

2.3 *Glassware*

Glassware and polyethylene cells that were used for solution preparations and measurements were soaked in an acid bath (1% HCl : 1% HNO_3) for one week. Also they were washed with detergent and then soaked in an acid bath (1% HCl : 1% HNO_3) overnight after use. Before they are used again each item was rinsed several times with fresh Mill-Q water.

2.4 *Pretreatment of electrode*

The platinum working-electrode was polished with 320 μM aluminium oxide, on a soft polishing pad to remove any previous film and then finally polished with 5 μm aluminium oxide. The electrode surface was thoroughly washed with Milli-Q water, rinsed under a stream of acetone and finaly 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.

2.5 *Preparation of bilayer electrode*

The preparation of the polypyrrole-based bilayer biosensor was a two-step process which involves the formation of an inner layer as one of the following:

2.5.1 Polypyrrole-chloride (PPy-Cl) film. The galvanostatic electropolymerization of the PPy-Cl film was performed 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 layer 1. The PPy-Cl film was formed in a monomer solution containing 0.4 M pyrrole and 0.1 M KCl, 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. It was shaken gently and the sides were dried with fibre-free tissue paper, to remove excess water. The electrode was then fixed to a retort stand in preparation for the next step.

2.5.2 Polypyrrole-nitrate (PPy-NO_3) film. The galvanostatic electropolymerization of the PPy-NO_3 film was prepared as described in section 2.5.1, except that the PPy-NO_3 film was formed in a monomer solution containing 0.4 M pyrrole and 0.1 M KNO_3.

2.5.3 Polypyrrole-KCl with ferrocyanide (PPy-Cl–$Fe(CN)_6^{4-}$). The galvanostatic electropolymerization of the PPy-Cl–$Fe(CN)_6^{4-}$ film was prepared as described in section 2.5.1, except that the monomer solution contained 0.4 M pyrrole, 0.1 M KCl and 20 mM $Fe(CN)_6^{4-}$.

2.5.4 Polypyrrole-nitrate with ferrocyanide film. The galvanostatic electropolymerization of the PPy-NO_3–$Fe(CN)_6^{4-}$ film was prepared as described in section 2.5.1, except that the monomer solution contained 0.4 M pyrrole and 0.1 M KNO_3 and 20 mM $Fe(CN)_6^{4-}$.

The formation of either 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 components listed in table 1 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.

Table 1. Composition of mixture used for layer 2 in PPy-NO_3–BSA–GLA–PNP–XOD biosensor.

Layer	Vol. BSA (μL)	Vol. GLA (μL)	Ratio XOD : PNP	Vol. mixture used (μL)
2	5 (6.8% w/v)	5 (4.5% v/v)	1 : 8 6.2 U mL^{-1} (XOD) 48.8 U mL^{-1} (PNP)	3

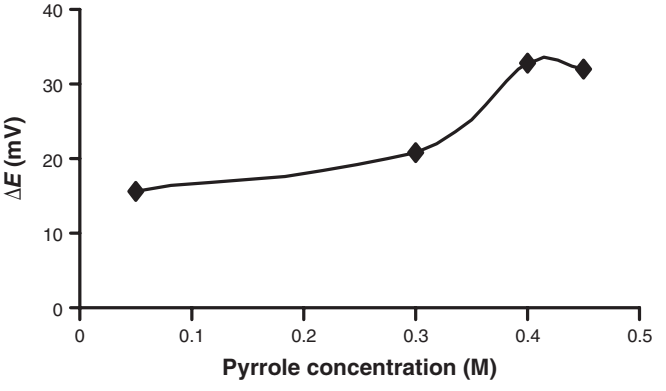

Figure 1. Effect of pyrrole concentration used for formation of film in layer 1 on the potentiometric response of phosphate obtained with bilayer PPy-NO_3/BSA–GLA–PNP–XOD. Concentrations of component in sensing layer were as described in table 1. Potentiometric measurement was made in 0.05 M Tris–HCl buffer and [phosphate] was 10 mM.

2.6 *Phosphate analysis in water samples*

The bilayer biosensor, 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 μL of raw water sample was added. When a steady-state potential was reached 100 μL 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 *Influence of pyrrole concentration and type of PPy film in layer 1 on sensitivity*

Figure 1 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 formation of thicker polypyrrole film which will increase the diffusion barrier.

Figure 2 and table 1 illustrate that irrespective of the type of pyrrole film used in the inner layer the response of the biosensor to phosphate was much lower at a polymerization time of 60 s than at 300 s. In fact, the sensitivity of the response

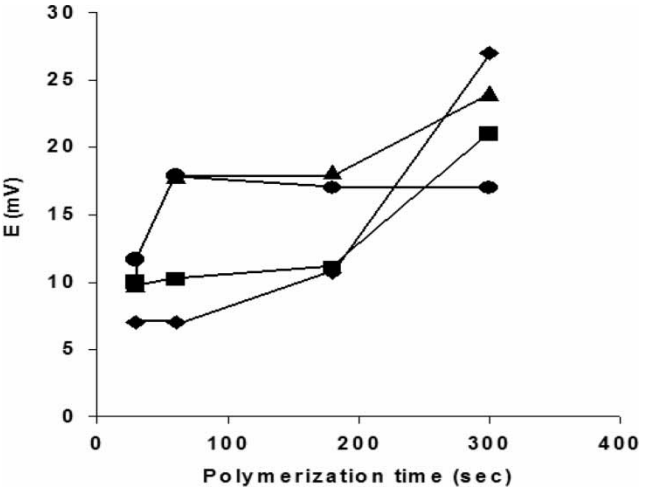

Figure 2. Effect of type of polypyrrole film in layer 1 on the potentiometric response of phosphate obtained with bilayer PPy-NO_3/BSA–GLA–PNP–XOD. [Phosphate] was 10 mM. ● PPy-Cl; ■ PPy-NO_3; ▲ PPy-Cl–$Fe(CN)_6^{4-}$; ◆ PPy-NO_3–$Fe(CN)_6^{4-}$.

obtained with the PPy-NO_3 films increased significantly with increasing polymerization time up to 300 s. In contrast, the response obtained with PPy-Cl inner layer seems to reach a maximum at 60 s. This may be due to the differences in the permeability of the product of the enzymatic reaction through the film, but in all cases the sensitivity was highest when polymerization was carried out for 300 s. A polymerization time of 300 s was therefore chosen for formation of the PPy-NO_3–$Fe(CN)_6^{4-}$ film, which gave the best sensitivity, as the inner layer in all subsequent work.

3.2 *Effect of pH, buffer concentration and drying time*

Figure 3(a) shows that the optimum phosphate response obtained with the bilayer PPy-NO_3/BSA–GLA–PNP–XOD electrode was at pH 7. D'Urso and Coulet [2, 3], as well as Guilbault and Nanjo [7] have previously reported that they obtained optimum phosphate response with biosensors based on the use of the same bienzyme system, but with different immobilization method. Evidently, the use of the bilayer arrangement in this work did not affect the optimum performance of the PNP–XOD bienzyme system.

Subsequently solutions buffered to pH 7.0 with Tris–HCl buffer were used for all other measurements. Figure 3(b) shows that increasing buffer concentration resulted in a decrease in the potentiometric response obtained for phosphate with the bilayer electrode. The use of the lowest buffer concentration of 0.025 M gave the optimum potentiometric response for phosphate. However, the establishment of the equilibrium potential at this concentration takes longer (around 25 min).

The drying of the outer layer is critical for obtaining reliable measurement with the bilayer PPy-NO_3/BSA–GLA–PNP–XOD electrode. Figure 4 shows that the phosphate response obtained with this electrode increased with the drying time up to 30 min. 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.

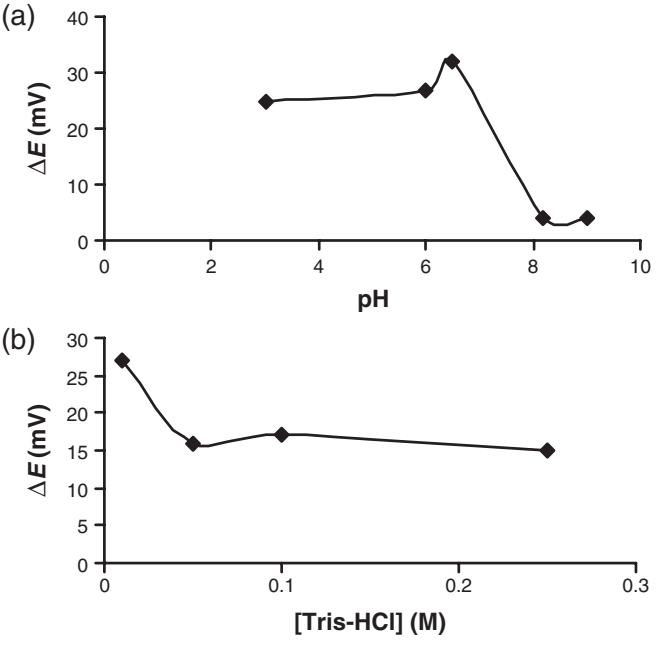

Figure 3. Effect of (a) pH and (b) buffer concentration on the potentiometric response of phosphate obtained with bilayer PPy-NO_3/BSA–GLA–PNP–XOD. [Phosphate] was 10 mM.

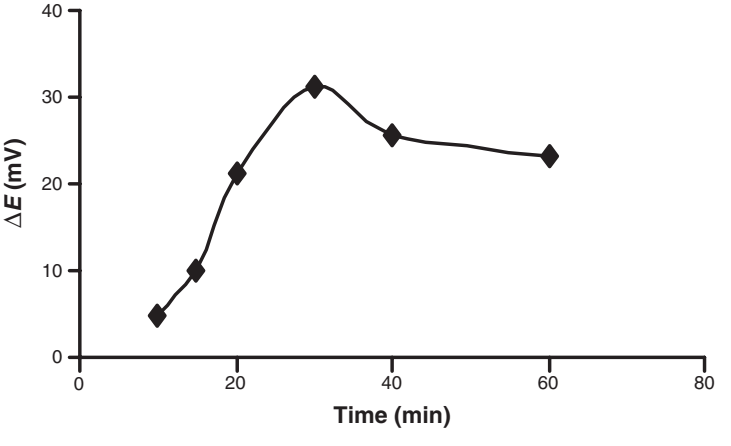

Figure 4. Effect of drying of pyrrole film in layer 2 on the potentiometric response of phosphate obtained with bilayer PPy-NO_3/BSA–GLA–PNP–XOD. [Phosphate] was 10 mM.

3.3 *Analytical applications*

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 for phosphate. This detectable amount is better than reported previously [4, 13, 17] with biosensors based on the use of the same bienzyme system. The achievable linear concentration range for phosphate

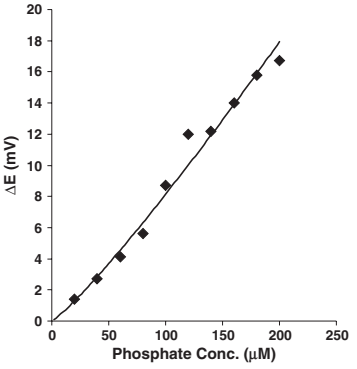

Figure 5. Calibration curve for phosphate on the PPy-NO_3/BSA–GLA–PNP–XOD electrode.

Table 2. Recovery of phosphate with bi-layer PPy–NO_3/BSA–GLA–PNP–XOD biosensor.

Phosphate added (μM)	Phosphate recovered (μM)	Percentage recovery (%)
25.3	24.9 ± 1.0	98.5 ± 1.9
50.6	50.8 ± 1.0	100.4 ± 2.4
101.2	101.7 ± 1.0	100.5 ± 3.8

Table 3. Recovery of phosphate in water samples.

Water sample	% Recovery
Lake	99.6 ± 3.0
Swamp	97.7 ± 2.5
River	98.8 ± 1.5

with this biosensor, as illustrated in figure 5, was between 20 and 200 μM phosphate. This linear range is wider than those obtained in our laboratories with single layer PPy–XOD–PNP and BSA–GLA–XOD–PNP biosensors. Evidently, the use of bilayer arrangement helps to extend the analytical applicability of the potentiometric biosensor for phosphate measurement. The addition of uric acid, ascorbic acid and glycine at 0–5 mM did not have any significant interference effect on the response.

Table 2 shows that excellent recoveries, in the range of 98.5–100.5%, were achieved with the bi-layer PPy-NO_3/BSA–GLA–PNP–XOD biosensor for the quantification of known spiked amounts of phosphate in deionized water. These results demonstrate that accurate and sensitive determination of phosphate can be accomplished with the biosensor in water samples that are free of interferants. The standard deviation ($n = 3$) values also demonstrate that the biosensor enables reproducible quantification. Table 3 also shows that excellent recoveries of phosphate in different spiked water samples were accomplished. Figure 6 shows a typical quantification of phosphate concentration in a lake water sample with the bi-layer PPy-NO_3/BSA–GLA–PNP–XOD biosensor by standard addition. 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 [18] that the potential of another

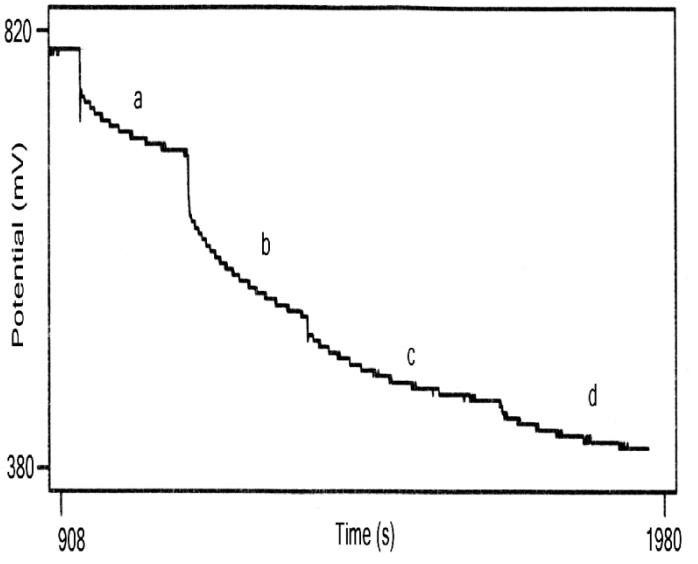

Figure 6. Typical responses obtained for the quantification of phosphate in Gippsland Lake with PPy-NO_3/BSA–GLA–XOD–PNP biosensor. Phosphate concentration (a) sample only, (b) 0.09, (c) 0.20 and (d) 0.29 mM.

polypyrrole biosensor decreases with increasing concentration of hydrogen peroxide and solution pH.

4. Conclusions

A PPy-NO_3/BSA–GLA–PNP–XOD bilayer biosensor has been successfully developed for phosphate determination. The minimum detectable amount of phosphate measured with the biosensor was 20 μM and a linear concentration range of 20–200 μM was achieved. Also, excellent recoveries (98.5–100.5%) of phosphate in lake, swamp and river water samples were obtained with the bilayer biosensor. The bilayer arrangement appeared to be useful in minimizing interference and increasing enzyme loading beyond the amount that can be achieved in a single layer polypyrrole biosensor.

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