Journal of Molecular Catalysis B: Enzymatic 63 (2010) 45–49

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

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

Polypyrrole-based potentiometric phosphate biosensor

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ARTICLE INFO

Article history:
Received 9 September 2009
Received in revised form 2 December 2009
Accepted 2 December 2009
Available online 6 January 2010

Keywords:
Polypyrrole
Biosensor
Xanthine oxidase
Purine nucleoxide phosphorylase
Conducting polymer

ABSTRACT

A phosphate biosensor based on potentiometric detection mode is described. Purine nucleoside phosphorylase (PNP) and xanthine oxidase (XOD) were immobilised into polypyrrole films for potentiometric measurement of phosphate in 0.05 M barbitone buffer (pH 7.8) which contain 10 mM inosine. A minimum detectable amount of 1.0 μM phosphate and a linear concentration range of 5–25 μM were achieved enabling sensitive potentiometric detection and a wide linear concentration range. The presence of uric and ascorbic acids had the least effect on the performance of the PPy–PNP–XOD–$Fe(CN)_6^{4-}$ biosensor and therefore will not have any effect on phosphate measurement at levels normally present in water.

1. Introduction

Phosphate ion determination methods are time-consuming and laborious often giving low sensitivity and poor stability [1–5]. An alternative is the use of a conducting polymer biosensor, which is able to measure the substrate directly in the sample [6–18]. With the advent of enzyme-based biosensors, several approaches have been investigated for detecting phosphate [6–12]. Most of the early works were based on alkaline or acid phosphatase with glucose [19–22], but later approaches that used the bienzyme system which includes PNP and XOD gained more interest [6–9,13,18,23,24]. Recently Rahaman et al. [12] developed a biosensor based on the pyruvate oxidase modified conducting polymer for phosphate ion determination. Pyruvate oxidase was also used by other researchers for the determination of phosphate ion [10,12,25–29]. A biosensor for inorganic phosphate using rhodamine labeled phosphate binding protein was developed by Okoh et al. [30] while another spectrophotometric method, based on a polyvinyl chloride matrix membrane sensor which was responsive to inorganic phosphate ion, was described by Lin et al. [31]. Other spectrophotometric methods based on molybdenum complex were described by Roger et al. [11], Galhardo and Masini [32], Fernandes and Reis [33], Nevesa et al. [34], Motomisu [35,36], and Mecozzi [37]. Those based on fluorescence and chemiluminescence were described by Gupta et al. [38], Lin et al. [31], Ikebukuro et al. [39], Nakamura et al. [40] and Yaqoob et al. [41]. Others used screen printed electrode [42] and conductometric methods [43].

With most of these electrochemical biosensors, the determination of phosphate concentration is accomplished amperometrically by monitoring the oxidation current of liberated H_2O_2 or the reduction current of oxygen consumed during the oxidation of hypoxanthine [Hx] enzymatic reaction.

To date, most of the reported phosphate biosensors detect phosphate by amperometric measurement of the liberated H_2O_2. In contrast to amperometric detection, potentiometric determination of phosphate requires a simpler construction of the phosphate biosensor and the use of a two-electrode system becomes feasible, in the analysis. The potentiometric response is generated as a result of the hydrogen peroxide produced from the electrode reaction. In this paper, the use of the potentiometric mode of detection is considered for biosensing of phosphate based on the enzymatic reaction shown in Eqs. (1) and (2) [44–46].

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

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

This will involve galvanostatic immobilisation of PNP and XOD into polypyrrole film. However, other factors have been considered in this case for improving the sensitivity of the potentiometric biosensor including:

(a) galvanostatic immobilisation conditions, such as pyrrole concentration, enzyme concentrations, magnitude of applied current density, and polymerisation time;
(b) measurement conditions such as pH and buffer concentration; and
(c) interferences from ascorbic acid, glycine and uric acid.

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doi:10.1016/j.molcatb.2009.12.002

2. Experimental

2.1. Reagents, chemicals and standard solutions

All chemicals were of analytical grade unless specified otherwise. Pyrrole was supplied by Aldrich (USA) and was distilled before use. The distilled pyrrole was stored in the refrigerator under a nitrogen atmosphere after covering the container with aluminium foil to prevent UV degradation and air oxidation. All solutions were prepared with Milli-Q water.

XOD (EC1.1.3.22 Grade 1) from buttermilk, purine nucleoside phosphorylase (EC2.4.2.1), inosine, and potassium ferrocyanide, were obtained from Sigma–Aldrich, Sydney, Australia. Other chemicals used were also of analytical grade, and all compounds used in this work were prepared without further purification. Phosphate stock solution (0.5 M) was stored in the refrigerator and was diluted when necessary to give the required standard concentration. A 0.05 M barbitone buffer (pH 7) was prepared by neutralising barbituric acid with sodium hydroxide.

2.2. Instrumentation

Electrochemical deposition of polypyrrole (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 the potentiometric and amperometric measurements. 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. Preparation of PNP/XOD enzyme electrode

Platinum disc electrodes were polished with 0.3 μm alumina on a polishing pad, rinsed with distilled water, acetone and once 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, XOD and PNP were immobilised into the polypyrrole film by electropolymerisation at various current densities and polymerisation times as described in our previous paper [47]. The PPy–PNP–XOD–$Fe(CN)_6^{4-}$ electrode obtained was rinsed carefully with the buffer. As no electron mediation is involved in the potentiometric measurement, the addition of $K_4Fe(CN)_6$ to the polymerisation solution was useful in improving the conductivity of the film.

The bienzyme system employed 6.2 U/mL of XOD to 49.6 U/mL of PNP, which corresponded to a 1:8 XOD to PNP ratio. Electropolymerisation was carried out galvanostatically in stagnant solutions in a three-electrode cell with Pt auxiliary and Ag/AgCl reference electrodes. The quantity of charge passed during the film formation was varied from 10 to 200 mC/cm^2 at different current densities (0.05–1.0 mA/cm^2) and electropolymerisation periods. The established conditions for growing the PPy–PNP–XOD– film are 0.5 M pyrrole (Py), 6.2 U/mL XOD, 49.6 U/mL PNP, a current density of 0.75 mA/cm^2, 20 mM $K_4Fe(CN)_6$ and an electrical charge of 150 mC/cm^2.

2.4. Potentiometric measurements

After electropolymerisation, the electrode was rinsed thoroughly with distilled 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 inosine. The resulting equilibrium potential vs. 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 response was tested by addition of known concentration into the cell prior to the potentiometric measurement.

3. Results and discussion

3.1. Optimisation of galvanostatic immobilisation conditions

PPy–PNP–XOD–$Fe(CN)_6^{4-}$ films are pH sensitive and it was found that the potential of the biosensor decreased with increasing phosphate concentration. This suggests that the resulting potentiometric response was due to hydrogen peroxide generation during the enzymatic reaction and possibly the sensitivity of the electroactive polymer layer to change in the pH of the solution. The potential difference developed may have originated from the redox couple of hydrogen peroxide produced. It has been shown in a previous study [15] that the potential of a polypyrrole-based glucose biosensor decreased with increasing concentration of glucose and solution pH.

The suitability of the biosensor to environmental use in terms of detection limit, stability and calibration range is strictly dependent on the enzyme loading. Fig. 1 shows that a ratio of 1:8 of XOD to PNP gave optimum potentiometric response. This is in agreement with the results obtained for amperometric detection by D'Urso et al. [6,45] who used BSA/GLA to immobilise PNP and XOD for amperometric measurement. Guilbault and Lubrano [48] found that a 1:10 ratio gave optimum response, while Kulys et al. [49] and Wollemberger et al. [13] obtained the best response with 1:5 ratio. Toshio Yao [50], using a reactor made of beads, found a ratio of 1:21 gave the best response, while Konisita et al. [51] optimised their biosensor with a 1:3 ratio.

The lower sensitivities when the XOD:PNP ratio was above 1:8 might be due to increase in film thickness which increases the diffusion barrier to hydrogen peroxide. At a lower XOD:PNP ratio (less than 1:8), the films were much thinner and the response may not have been due to the conductivity changes in the pyrrole film, but may have resulted mainly from the response of the bare electrode to phosphate ion [52]. The optimum response was obtained with films formed with 0.5 M pyrrole. At a lower concentration the sensitivity of the response was low and this could have been due to inadequate coverage of the electrode and insufficient entrapment

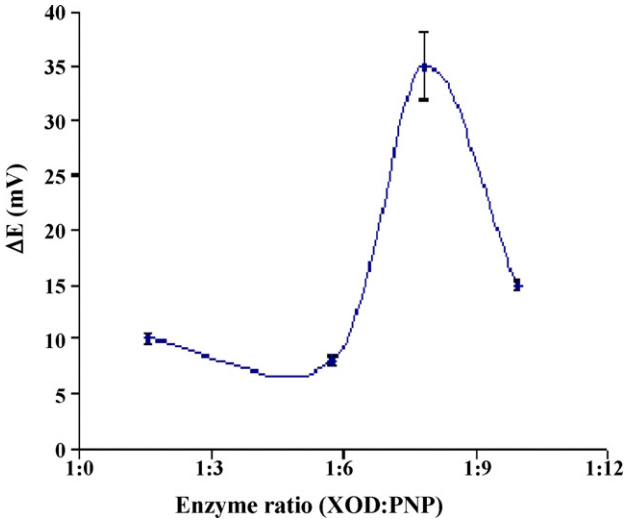

Fig. 1. Effect of XOD:PNP ratio on phosphate response in 0.05 M barbitone buffer (pH 7.8). The amount of XOD was kept constant. [Phosphate] was 10 mM.

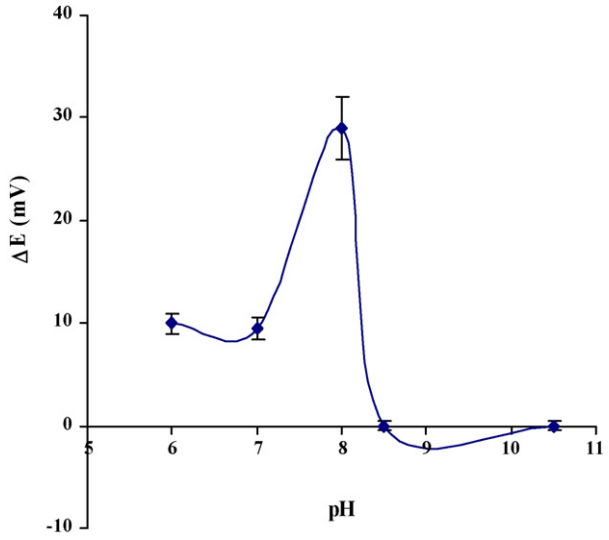

Fig. 2. Effect of varying pH on phosphate response obtained with PPy–PNP–XOD–$Fe(CN)_6^{4-}$ biosensor. Potentiometric measurement was made in 0.05 M barbitone buffer. [Phosphate] was 10 mM.

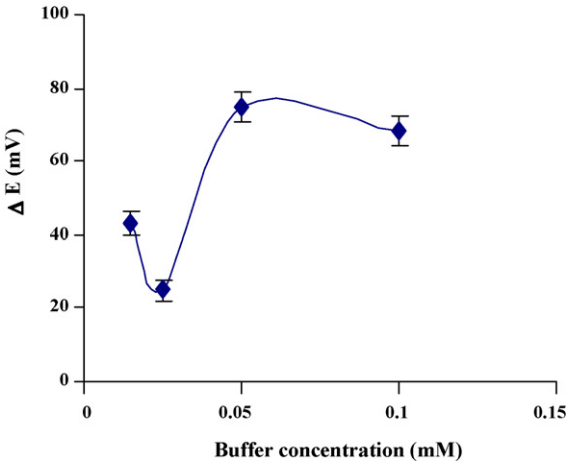

Fig. 3. Effect of varying buffer concentration on the phosphate response obtained with PPy–PNP–XOD–$Fe(CN)_6^{4-}$ biosensor. Potentiometric measurement was made in 0.05 M barbitone buffer. [Phosphate] was 10 mM.

of PNP and XOD enzymes. Above the optimum concentration, the response decreased and this could have been due to the increase in the thickness of the PPy–PNP–XOD–$(FeCN)_6^{4-}$ film, which resulted in increased diffusion barrier.

The response time is affected by the enzymatic reaction, the permeability of the substrate, and the diffusion rate of hydrogen peroxide through the polypyrrole film. If the film is thick, the permeability of the substrate and the diffusion rate of H_2O_2 are lower, resulting in a longer response time. The thickness of the polypyrrole film formed was related to the amount of charge passed during film formation and the higher the charge the thicker the film in accordance with the results reported by Holdcroft and Funt [58]. In general, the thicker films are less sensitive than the thinner films [52]. The best potentiometric response was obtained with film formed for a polymerisation period of 200 s. A lower polymerisation period resulted in an inadequate coverage of platinum and resulted in lower sensitivity. In contrast, a polymerisation period higher than 200 s gave adequate coverage, but the response sensitivity was lower due to the increased diffusion barrier caused by the increased film thickness.

The charge passed during electropolymerisation corresponds to the film thickness and the response time of the PPy–PNP–XOD–$(FeCN)_6^{4-}$ electrode to phosphate is influenced by the film thickness. This is because more time is required for the product of the enzymatic reaction of phosphate ion to penetrate into the thicker film and to reach the surface of the Pt electrode. PPy–PNP–XOD–$(FeCN)_6^{4-}$ biosensor was not sensitive to phosphate when the charge passed during electropolymerisation exceeded 150 mC/cm^2.

Therefore, the optimum conditions achieved in this study for obtaining sensitive film of PPy–PNP–XOD–$(FeCN)_6^{4-}$ were 0.5 M Py, 6.2 U/mL XOD and 49.6 U/mL. PNP, an applied current density of 0.75 mA/cm^2, 20 mM $K_4Fe(CN)_6$ and a charge of 150 mC/cm^2. Based on the assumptions [15,19] that a charge of 45 mC/cm^2 produces a film thickness of 0.1 μm [15,58], the thickness of the PPy–PNP–XOD–$Fe(CN)_6^{4-}$ film formed with a charge of 150 mC/cm^2 was about 0.33 μm or 330 nm.

3.2. Optimisation of potentiometric measurement conditions

Fig. 2 shows the effect of pH on the response of the PPy–PNP–XOD–$Fe(CN)_6^{4-}$ electrode. The optimum potentiometric response for phosphate was obtained at pH 7.8 close to the optimum pH of the respective enzyme in solution [53]. Guilbault [54] and Kulys et al. [49] found that pH 7 gave the optimum sensitivity for a phosphate biosensor based on the immobilisation of the same enzymes with BSA/GLA, while D'Urso [6,45] and Yao et al. [50] found pH 7.5 to be ideal for their biosensor which was formed by immobilisation of the enzymes on beads bound covalently with GLA. The optimum pH of 7.8 obtained in this study was used for all other measurements.

No response to phosphate was obtained when the buffer or sample solution did not contain inosine, which had to be supplied in excess in a phosphate-free buffer. Barbitone buffer was chosen for these experiments and 5 mM inosine was found to be sufficient. Wollemberger et al. [13] also used excess inosine (>5 mM) to ensure a co-reactant independent phosphate response. The addition of 5 mM of inosine gave the optimum response for phosphate.

As PNP catalyses the phosphorylysis of inosine to ribose 1-phosphate and Hx, the resulting hypoxanthine is oxidised by XOD, as shown in Eqs. (1) and (2). The H_2O_2 and uric acid produced are electrochemically active and therefore can be easily detected. Uric acid can also cause a response due to a change in pH. Any change in pH of the film could result in a change in the reaction speed. Optimisation of the buffer concentration is therefore necessary and needed to be considered in this section. Fig. 3 shows the effect of buffer concentration on the potentiometric response for phosphate. The increasing buffer concentration resulted initially in the decrease in the response and then increased with increasing buffer concentration up to 0.05 M. 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 affects the magnitude of the response. The optimum buffer concentration of 0.05 M was used for all subsequent measurements.

3.3. Interference study

Most natural raw water samples and biological materials have substances that may be electroactive [19]. In particular, it has been reported that some organic acids (ascorbic acid, uric acid and some oxidisable organic materials (glycine)) interfere with the determination of phosphate [55]. The specificity of a biosensor against these interferences is therefore of paramount importance because it reduces the need for pre-treatment, such

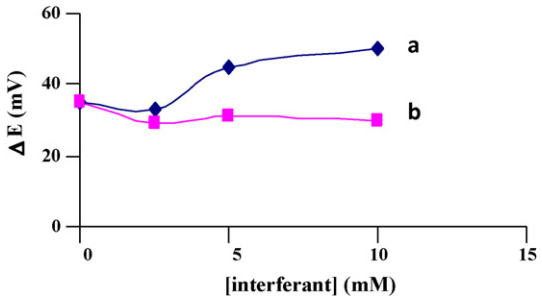

Fig. 4. Effect of ascorbic acid (a) and uric acid (b) on phosphate response obtained with PPy–PNP–XOD–$Fe(CN)_6^{4-}$ biosensor. Potentiometric measurement was made in 0.05 M barbitone buffer. [Phosphate] was 10 mM.

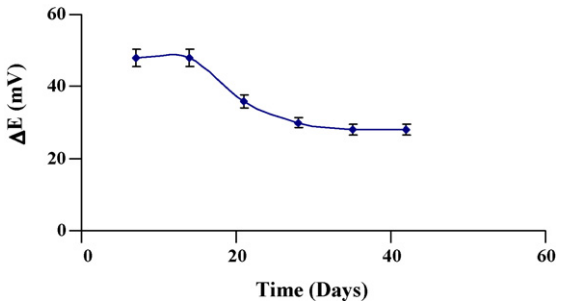

Fig. 5. Influence of storage time on the sensitivity of phosphate response obtained with potentiometric PPy–PNP–XOD–$Fe(CN)_6^{4-}$ biosensor.

as separation [56], and makes the biosensor more amenable to automated analysis. Thus, biosensors that are used should be specific enough to discriminate against these coexisting substances [19,56]. Alternatively, other approaches that suppress the interferences, such as the modification of working electrodes with polyphenol, can be used. It has been shown that polyphenol films provide some permselectivity at the electrode surface [19]. In this study, the effect of three interfering species frequently found in biological and water samples [19,56,57] was considered. It was observed that the presence of up to 2.5 mM ascorbic acid did not interfere with the potentiometric response of the biosensor to phosphate. However, ascorbic acid concentration greater than 2.5 mM enhanced the response by up to 42%. The observed enhancement could be due to the penetration of ascorbic acid through the PPy film to the electrode surface and subsequent increase in potentiometric signal as shown in Fig. 4(a). Table 1 shows the difference between potentiometric response at zero interferant and at different interferant concentrations. Similar enhancement effects have been reported for phosphate and glucose biosensors constructed by different immobilisation methods [15,57].

It was observed that in the presence of 2.5 mM uric acid the change in potential was suppressed by 17% as shown in Fig. 4(b). This confirms a previous report that the presence of uric acid reduced the response of the enzyme biosensor [19]. Uric acid is a major interferant to most biosensors. It is a common interferant, but it is not as limiting as ascorbic acid. 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 the phosphate measurements [19].

Glycine did not interfere with the biosensor response to phosphate. Glycine, being a protein, may adsorb onto a polypyrrole/platinum electrode surface and interferes by blocking the surface of the polymer [19]. However, the presence of glycine in water or other aqueous solutions did not interfere with the biosensor response to phosphate and it is probable that other proteins would not interfere either.

Table 1
Effect of ascorbic acid concentration on phosphate response obtained with PPy–PNP–XOD–$Fe(CN)_6^{4-}$ biosensor.

Ascorbic acid (mM)	Change in potential (mV)	Percentage of response enhancement (%)
0	35	0
2.5	33	5.7
5	45	28.6
10	50	42.8
50	50	42.8

3.4. Stability of biosensor response

Fig. 5 shows that the phosphate response obtained with the PPy–PNP–XOD–$Fe(CN)_6^{4-}$ decreased slowly by 20% of its initial value after 24 h. In general, the PPy–PNP–XOD–$Fe(CN)_6^{4-}$ electrode lost more than 20% of its sensitivity per day if it was not stored in barbitone buffer at pH between 7 and 7.8. The sensitivity of the biosensor decreased rapidly for the first 2 days and at 8th day it had reached 50% of its initial value. Beyond 12 days it continued to decrease and eventually stabilised at about 20% of its initial value after 2 weeks. The observed decrease in sensitivity of the biosensor may be attributed to the loss of enzyme and ferrocyanide into the bulk solution.

3.5. Linear calibration and minimum detectable concentration

Fig. 6 shows the linear calibration curve obtained with the PPy–PNP–XOD–$Fe(CN)_6^{4-}$ electrode under optimised conditions. The response of this electrode in potentiometric mode was linear for phosphate concentration in the range 5–25 μM. The regression equation obtained was $y = 0.517x + 3.07$, correlation coefficient was 0.96 and the log plot has a correlation coefficient of 0.98. This range is lower than linear range of 0.1–1 mM obtained in amperometric mode [59]. An average correlative variance of all triplet potentiometric response in calibration curve was approximately 3.1%. A possible reason why changes in potential are linear with phosphate concentrations instead of Nernstian is possibly due to the complex nature of the composite electrode and the extent to which the Nernstian behaviour is maintained, which will be dependent on various parameters such as enzyme loading, film thickness and substrate (phosphate ion) concentration.

The minimum detectable concentration (MDC) of phosphate with the PPy–PNP–XOD–$Fe(CN)_6^{4-}$ biosensor in potentiometric mode was 1 μM which is much lower than 10 μM obtained with PPy–PNP–XOD–$Fe(CN)_6^{4-}$ in amperometric mode [60]. This is also better than the minimum detectable concentration of 20 μM

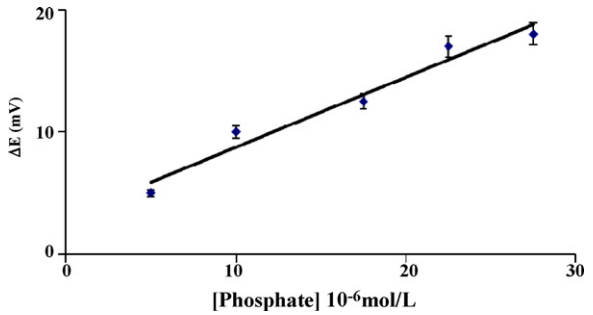

Fig. 6. Calibration curve for phosphate obtained with PPy–PNP–XOD–$(FeCN)_6^{4-}$ biosensor ($n = 5$).

obtained by other researchers [12,51]. Ikebukuro et al. [28,39] also reported a detection limit of 3.2 μM which is not as good as the MDC achieved in this study. However, this was not sufficient for analysis of phosphate in river water because the maximum permissible concentration of phosphate in river water suitable for drinking is 0.05 μM in Australia and New Zealand [60].

4. Conclusions

A PPy–PNP–XOD–$Fe(CN)_6^{4-}$ biosensor has been fabricated for accurate potentiometric measurements of phosphate at concentrations that are suitable for environmental monitoring. It can detect a minimum of 1.0 μM phosphate ion and has a linear concentration range of 5–25 μM. The device appears promising for analysing phosphate in polluted water. It was observed that ascorbic acid concentration greater than 2.5 mM enhanced the response by up to 42%, while in the presence of 2.5 mM uric acid the change in potential was suppressed by 17%.

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