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Fabrication of a bilayer potentiometric phosphate biosensor by cross-link immobilization with bovine serum albumin and glutaraldehyde

Samuel B. Adeloju*, Abdulazeez T. Lawal

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

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ABSTRACT

Chemical cross-linking of purine nucleoside phosphorylase (PNP) and xanthine oxidase (XOD) with glutaraldehyde (GLA) and bovine serum albumin (BSA) has been used to fabricate a stable and reliable bilayer potentiometric phosphate biosensor. The bilayer arrangement consists of an inner BSA–GLA layer and an outer BSA–GLA–PNP–XOD layer. The inclusion of the inner BSA–GLA layer improves the adhesion of the outer BSA–GLA–PNP–XOD layer and ensures stability of the phosphate biosensor. Established optimum conditions for immobilization of the enzymes in the outer layer and for reliable potentiometric measurement were 4.5% v/v GLA, 6.8% w/v BSA, XOD:PNP mole ratio of 1:8, and a film drying time of 30 min. As little as 20 μM of phosphate can be detected with the BSA–GLA/BSA–GLA–XOD–PNP bilayer biosensor with a linear concentration range between 40 and 120 μM. The biosensor was very stable for 21 days, achieving a good reproducibility with a rsd of only 5.7% and, even after more than a month, the change in the initial potential value was only 10%.

1. Introduction

The electrochemical biosensing of phosphate often rely on the use of various enzyme immobilization methods [1–16]. In particular, methods such as adsorption [2,17], covalent bonding [18], entrapment [1] and cross-linking [19] are commonly employed. Notably, cross-linking has attracted considerable interests because of its simplicity for direct immobilization of enzymes, such as xanthine oxidase (XOD) and purine nucleoside phosphorylase (PNP), on various electrodes [19–23]. Other enzymes that have been used for fabrication of phosphate biosensors include pyruvate oxidase [18,24–29], alkaline phosphatase [30], acid phosphatase, maltose phosphorylase [15,31] and glucose oxidase [12]. The determination of phosphate has also been accomplished by use of several analytical methods, such as spectrophotometry based on formation of molybdenum complex [6,32–39], fluorescence [40,41], chemiluminescence [26,31,42–46], screen printed electrodes [29,47] and conductometry [43]. Of all of these approaches, the use of PNP–XOD bienzyme system is particularly advantageous for improving sensitivity of phosphate biosensors and has attracted a lot of interest.

The basis for electrochemical biosensing of phosphate with the PNP–XOD bienzyme system involves initial conversion of orthophosphate in the presence of inosine to hypoxanthine:

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

Followed by XOD-catalysed oxidation of the resulting hypoxanthine to H_2O_2 [5–7,9,10]:

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

The distinct advantage of using the PNP–XOD bienzyme system lies in its ability to produce a higher amount of hypoxanthine, H_2O_2 and, hence, to give a higher sensitivity than other enzyme-based phosphate biosensors [9,21–23,48,49]. In most cases, the measurement of phosphate with these biosensors is usually accomplished by amperometric detection of H_2O_2 or the oxygen consumed during the enzymatic reaction. However, in a recent study [50], we demonstrated that a two electrode system can be used with enzyme entrapment in polypyrrole film for constructing a simpler biosensor for potentiometric detection of phosphate. The potential change caused by the H_2O_2 produced during the catalytic reaction was measured and related to phosphate concentration. Unfortunately, the stability of the PPy-based potentiometric biosensor was poor [50,51]. Within 8 days, the initial potentiometric response obtained for phosphate with this biosensor dropped by 50% and decreased even further to remain at 20% of the initial response after 2 weeks [50]. There is therefore still a need for development of other appropriate

* Corresponding author. Tel.: +61 399026450; fax: +61 399026738.
E-mail address: Sam.Adeloju@monash.edu (S.B. Adeloju).

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strategies for improving the stability of potentiometric phosphate biosensor.

Although BSA and GLA have been used in other studies [19–23] for fabrication of phosphate biosensors, it is more commonly employed as a wash solution or in a single layer arrangement which does not lend itself to long-term stability. In this study, we investigate the possibility of using BSA and GLA in a bilayer arrangement for chemical cross-link immobilization of PNP and XOD and, hence, for achieving reliable and stable potentiometric detection of phosphate. Factors considered for achieving efficient chemical cross-linking of PNP and XOD include PNP:XOD ratio, GLA and BSA concentrations, pH and buffer concentrations. Also, the influence of the drying time for formation of the BSA–GLA film on the performance of the biosensor is investigated. Furthermore, a comparison is made between the performance of the resulting BSA–GLA/BSA–GLA–XOD–PNP bilayer biosensor and that of a PPy-based phosphate potentiometric biosensor.

2. Experimental

2.1. Chemicals and standard solutions

XOD (EC1.1.3.22 Grade 1) from buttermilk, PNP (EC2.4.2.1), and inosine were obtained from Sigma–Aldrich, Sydney, Australia. GLA (25% v/v aqueous solution) and BSA were also purchased from the Sigma–Aldrich Chemical Company. Stock solutions of 15% w/v BSA and 10% v/v GLA were prepared and stored in the refrigerator at −4 °C. These were subsequently diluted to give appropriate concentrations. The GLA stock solution concentration was deliberately made lower than that of BSA stock solution in the mixture used for formation of the layers to avoid excess cross-linking and, hence, to prevent denaturation of PNP and XOD. Stock solution (0.1 M) of Tris–HCl was prepared and diluted as required. NaCl (0.1 M) was added to the buffer solution during potentiometric measurement.

2.2. Procedures

2.2.1. Enzyme immobilization

The bilayer potentiometric biosensor was fabricated by a two-step procedure. For the inner layer, 1 μL of a mixture of 6.8% w/v BSA and 4.5% v/v GLA was applied to a platinum electrode at room temperature and allowed to air dry for at least 30 s (when the mixture has gelatinised and hardened). This inner BSA–GLA layer was deliberately applied to improve the adhesion of the outer BSA–GLA–PNP–XOD layer. Once the inner layer has dried, 3 μL of a mixture which contained 5 μL of 6.8% w/v BSA, 5 μL of 4.5% v/v GLA, 6.2 U mL^{-1} XOD, and 49.6 U mL^{-1} PNP was spread on top of the inner layer. This layer was then left to dry for at least 15 min to ensure adequate sensitivity for phosphate detection. Prior to use, the electrode was washed under a stream of Milli-Q water to remove any loosely bound molecules. The influence of the film drying time on the sensitivity of phosphate potentiometric response was also investigated.

2.2.2. Potentiometric measurement

All measurements were made in a two-electrode cell which contained 10 mL of Tris–HCl buffer solution (pH 7.0) into which 10 mM inosine was added, and the solution was stirred continuously. The chosen inosine concentration was based on previously reported optimum concentration for phosphate response [51]. Prior to addition of standard phosphate solution into the cell, the electrode potential was allowed to stabilize for at least 15 min. The potentiometric response of the sensor was measured versus Ag/AgCl electrode after each addition of standard phosphate solution to the cell. The minimum detectable concentration was determined from the smallest addition of standard phosphate solution which gave a phosphate response.

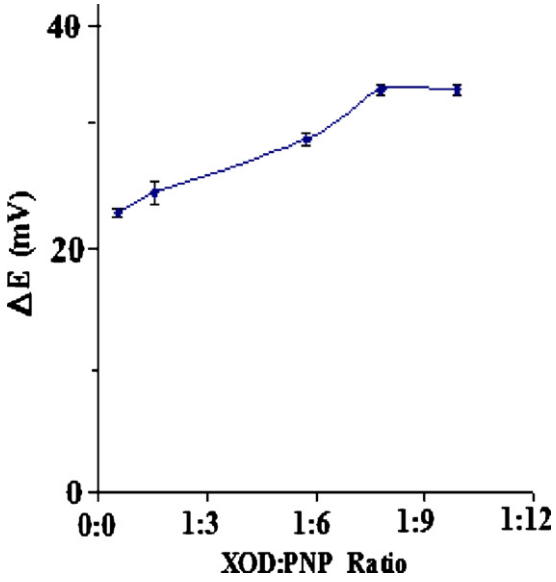

Fig. 1. Influence of varying PNP:XOD ratio on phosphate potentiometric response obtained with BSA–GLA/BSA–GLA–PNP–XOD bilayer biosensor. Potentiometric measurement was made in 0.05 M Tris–HCl buffer which contained 10 mM inosine; [Phosphate] was 10 mM; $n = 4$.

3. Results and discussion

3.1. Potentiometric detection of phosphate with BSA–GLA/BSA–GLA–PNP–XOD bilayer electrode

Based on the reactions given in Eqs. (1) and (2), the phosphorylation of inosine to ribose-1-phosphate and hypoxanthine (Hx) was catalysed initially by PNP, followed by the catalysis of oxidation of Hx by XOD in the presence of molecular oxygen to produce hydrogen peroxide which was detected by the BSA–GLA/BSA–GLA–XOD–PNP bilayer electrode. The detection of phosphate by this electrode can be significantly influenced by factors such as PNP:XOD ratio, BSA concentration, GLA concentration, drying time, pH, and buffer concentration. The influences of these parameters on the phosphate potentiometric response obtained with the bilayer BSA–GLA/BSA–GLA–XOD–PNP electrode are discussed below.

3.2. Influence of XOD:PNP ratio on phosphate response

The enzyme loading in the outer BSA–GLA layer can significantly influence the achievable detection limit, stability and calibration range achieved with the BSA–GLA/BSA–GLA–XOD–PNP electrode. As shown in Fig. 1, the phosphate potentiometric response obtained with this electrode increased with increasing PNP concentration in the XOD:PNP ratio incorporated in the outer layer. Evidently, the phosphate potentiometric response increased with increasing mole ratio up to 1:8 and remained constant at higher mole ratios. It is obvious from these results that the incorporation of XOD and PNP at a mole ratio ≥1:8 of XOD:PNP gave the most sensitive phosphate potentiometric response. This corresponds to the use of 6.2 U mL^{-1} XOD and 49.6 U mL^{-1} PNP in the mixture used for formation of the BSA–GLA–XOD–PNP outer layer. This is in agreement with a 1:8 mole ratio reported previously for amperometric detection of phosphate [21,22,50,51]. The resulting phosphate potentiometric responses were reasonably reproducible, resulting in a relative standard deviation ($n = 4$) of 5.8%. Consequently, a 1:8 mole ratio of

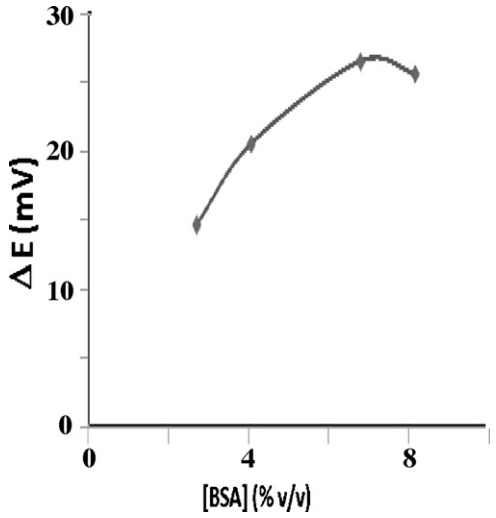

Fig. 2. Influence of varying [BSA] on phosphate potentiometric response obtained with the BSA–GLA/BSA–GLA–PNP–XOD bilayer biosensor. Potentiometric measurement was made in 0.05 M Tris–HCl buffer which contained 10 mM inosine; [Phosphate] was 10 mM; XOD:PNP ratio was 1:8 and GLA was 4.8% v/v. $n = 4$.

XOD:PNP was used for formation of all BSA–GLA–PNP–XOD outer layer.

3.3. Influence of BSA concentration on phosphate response

BSA is a lysine-rich auxiliary protein, mainly used in chemical crosslinking and it is functionally inert. The inclusion of BSA with GLA is useful in reducing the porosity of the film thereby making it more stable and, thus, increasing the responsiveness of the film [24]. It also increases the total protein concentration, allowing gel formation from solutions that would otherwise give only soluble oligomers. As shown in Fig. 2, the use of 2.4% w/v BSA for formation of the BSA–GLA–PNP–XOD outer layer gave low phosphate potentiometric response, but increased to an optimum with increasing BSA concentration up to 6.8% w/v. This may be due to insufficient spacing between PNP/XOD and GLA when low concentration of BSA is present. It has been reported that close proximity problems, which can be caused by cross-linking a single enzyme at a lower BSA concentration, is minimised because BSA is a spacer [18,19]. For this reason, 6.8% w/v of BSA was used to ensure optimum phosphate response and a good reproducibility with a rsd ($n = 4$) of 5.7%. The use of BSA concentrations >6.8% w/v resulted in lower response and the film did not have good mechanical properties. A decrease in enzyme activity may also occur at the higher BSA concentrations as the protein content becomes high, resulting in the spacing of the enzyme molecules too far apart.

3.4. Influence of GLA concentration

PNP and XOD were immobilized in GLA, a cross-linking reagent, which links the protein molecules together and forms an insoluble gel-matrix [52]. To avoid excess cross linking which could cause denaturation of the enzyme [15], it is important to ensure that the GLA concentration is lower than the BSA concentration. The results in Fig. 3 show that the optimum phosphate potentiometric response was obtained when 4.8% v/v of GLA was used for formation of the BSA–GLA–PNP–XOD outer layer. Also at this GLA concentration, the phosphate potentiometric responses obtained with films formed were reproducible with a rsd ($n = 4$) of 5.7%. At GLA concentration below this value (<4.8% v/v), the colour of the films produced was opaque light yellow as opposed to the dark yellowish colour film obtained at the optimum GLA concentration.

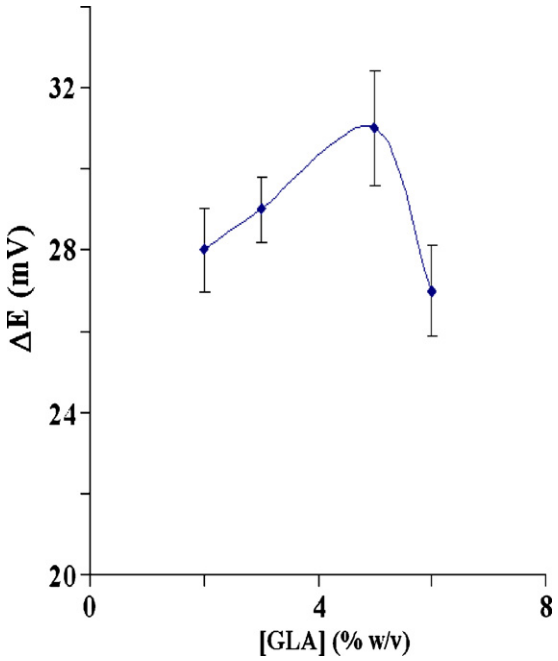

Fig. 3. Influence of varying [GLA] on phosphate potentiometric response obtained with the BSA–GLA/BSA–GLA–PNP–XOD bilayer biosensor. Potentiometric measurement was made in 0.05 M Tris–HCl buffer which contained 10 mM inosine; [Phosphate] was 10 mM; XOD:PNP ratio was 1:8; and BSA was 6.8% w/v. $n = 4$.

This observation suggests that the lower GLA concentration was not enough to allow adequate cross-linking of PNP and XOD in the outer layer and, hence, accounts for the lower potentiometric responses. As can be expected, adequate diffusion control and a good reaction rate cannot be achieved if insufficient amounts of PNP and XOD are immobilized and this will result in a decrease in the biosensor response [15]. The use of GLA concentration >4.8% v/v resulted in a considerable decrease in the potentiometric response. Chemical alterations of the catalytic sites of the protein [53], change in porosity, and the denaturing effect of excess GLA on XOD and PNP are some possible reasons for the observed decrease in the potentiometric response. When enzymes are cross-linked directly to GLA they tend to lose activity and the extent of the deleterious effect is dependent on the nature of the enzyme [54–56]. The observed decrease in phosphate potentiometric response obtained with outer layers formed with >4.8% v/v GLA may be due to the loss of activity and limitation of substrate diffusion caused by the change in physical structure of the film, such as an increase in the film thickness, which increases the diffusion barrier. This, in turn, reduces the amount of the catalytic product that can reach the electrode surface and, consequently, resulting in a reduction in the biosensor sensitivity [24,34,35]. For free movement of substrate and product, as well as a good rate of reaction with immobilized enzymes, it is important that the sensors have good diffusion properties [24]. When there is large enzyme activity within a thin enzyme layer, an effective external mass transfer is provided and the highest sensitivities are achieved [24]. For these reason, a GLA concentration of 4.8% v/v was used for formation of BSA–GLA–PNP–XOD outer layer for all other potentiometric measurements.

3.5. Optimisation of drying time

Fig. 4 shows the potentiometric response obtained for phosphate with the BSA–GLA–XOD–PNP outer layer formed with increasing drying time. Evidently, the potentiometric response increased with drying time up to 30 min. The outer layer left to dry for only 10 min was found to be slightly watery in appear-

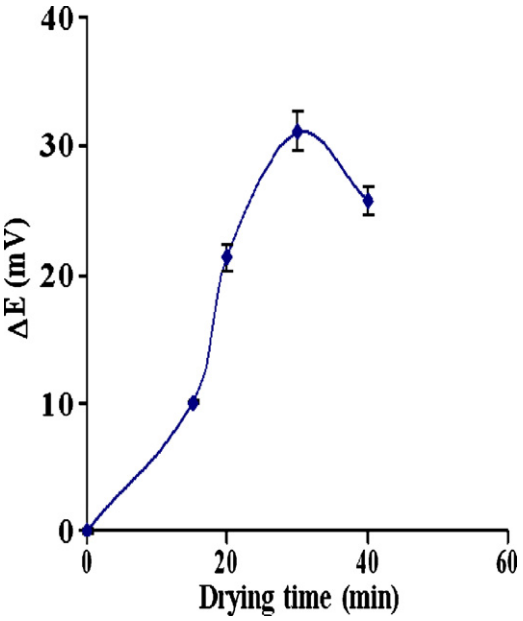

Fig. 4. Influence of varying drying time on phosphate potentiometric response obtained with the BSA–GLA/BSA–GLA–PNP–XOD bilayer biosensor. Potentiometric measurement was made in 0.05 M Tris–HCl buffer which contained 10 mM inosine; [Phosphate] was 10 mM; XOD:PNP ratio was 1:8; BSA was 6.8% w/v; GLA was 4.8% v/v; $n = 4$.

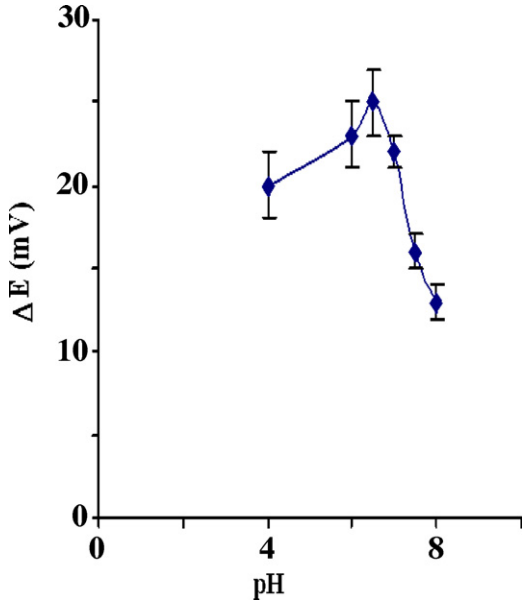

Fig. 5. Influence of varying pH on phosphate potentiometric response obtained with the BSA–GLA–PNP–XOD biosensor. Potentiometric measurement was made in 0.05 M Tris–HCl buffer which contained 10 mM inosine; [Phosphate] was 10 mM; XOD:PNP ratio was 1:8; BSA was 6.8% w/v; GLA was 4.8% v/v; drying time was 30 min; $n = 4$.

ance due to insufficient time for the GLA to polymerise. It was obvious therefore in this case that the PNP and XOD will not be adequately immobilized due to incomplete cross-linking. Consequently, only outer layers dried for at least 15 min can be used for reliable phosphate potentiometric measurements. A drying time of 30 min gave optimum phosphate potentiometric response. The use of longer drying times did not improve the sensitivity of the phosphate response. Excess cross-linking of XOD and PNP [1,57] may occur with longer drying times and this may be responsible for the poor diffusion properties and lack of effective external mass transfer. The polymer matrix and the resulting sensitivity can be affected when the composition of GLA changes over time as it polymerises at room temperature. A drying time of 30 min was therefore used for formation of the BSA–GLA–PNP–XOD outer layer for all other potentiometric measurements.

3.6. Influence of pH and buffer concentration

Fig. 5 shows that the phosphate potentiometric response obtained with the BSA–GLA/BSA–GLA–XOD–PNP bilayer biosensor increased with increasing pH of the buffer used for the measurement up to pH 6.8. Similar pH has been cited previously as optimum for detection of phosphate with PNP and XOD [8], but was lower than pH 7–7.8 reported by other researchers [12,58,59]. The lower pH obtained in this study may be due to the immobilization of XOD and PNP in the BSA–GLA mixture. Consequently, a Tris–HCl buffer of pH 6.8 was used for all other investigations.

More interestingly, the phosphate potentiometric response obtained with the BSA–GLA/BSA–GLA–XOD–PNP bilayer biosensor was significantly influenced by buffer concentration, as illustrated in Fig. 6. The phosphate response decreased with increasing buffer concentration, indicating that the potentiometric detection of catalytic process of the biosensor was hindered by the increasing buffering capacity of more concentrated buffer solutions. Under the high ionic strength of the more concentrated buffer solutions, the movement of H_2O_2 to the electrode surface becomes restricted and, consequently, results in lower potentiometric response. Alternatively, the observed lower phosphate sensitivity may be due to the lowering of phosphate activity under conditions of high ionic strength. Similar reduction in potentiometric response of a glucose biosensor at higher buffer concentration has been previously reported [60].

3.7. Analytical performance and stability

Increasing addition of phosphate resulted in increases in phosphate potentiometric response obtained with the BSA–GLA/BSA–GLA–XOD–PNP bilayer biosensor, but the response did not increase much beyond 100 μM phosphate and, thus, resulted in a non-linear curve. However, as expected, a plot of potential versus logarithm of phosphate concentration

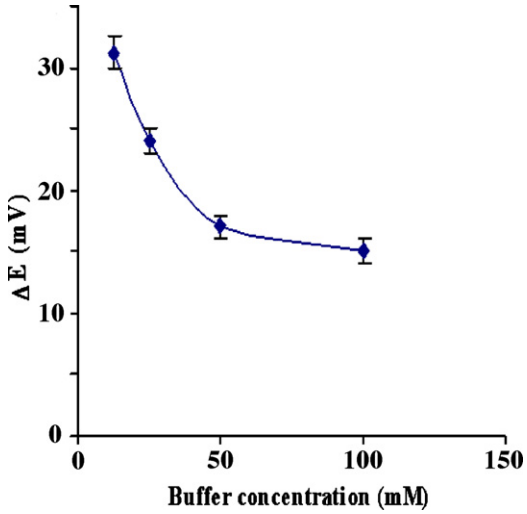

Fig. 6. Influence of varying buffer concentration on phosphate potentiometric response obtained with the BSA–GLA–PNP–XOD electrode. Potentiometric measurement was made in Tris–HCl buffer which contained 10 mM inosine; [Phosphate] was 10 mM; XOD:PNP ratio was 1:8; BSA was 6.8% w/v; GLA was 4.8% v/v; drying time was 30 min; $n = 4$.

Table 1
Optimum conditions established for phosphate measurement with the BSA–GLA/BSA–GLA–XOD–PNP bilayer biosensor.

Parameters	Abbreviation	Optimum condition
BSA concentration	[BSA]	6.8% w/v
GLA concentration	[GLA]	4.8% v/v
Ratio of XOD to PNP	XOD:PNP	1:8
Air-dry time	t	30 min
Minimum detectable concentration	MDC	20 μM
Linear concentration range	LCR	40–100 μM (direct plot) 40–120 μM (log plot)

gave a linear curve between 20 and 200 μM with a slope of 46.5 ± 1.0 mV decade^{-1}. This slope value was not close to the 29.5 mV decade^{-1} expected for a two-electron process, but was in agreement with a slope value of 45.5 mV decade^{-1} reported previously for a phosphate biosensor [3,13,61]. The observed deviation in the slope may be due to the nature of the electrode material and its method of preparation, including the enzyme loading, film thickness and substrate (phosphate ion) concentration.

The minimum detectable phosphate concentration achieved with the BSA–GLA–XOD–PNP biosensor was 20 μM, which is significantly lower than 50 μM reported previously [4] and is similar to those reported by others [11,18]. Evidently, the use of BSA–GLA mixture was effective for immobilization of PNP and XOD for potentiometric detection of phosphate. Nevertheless, further improvement in sensitivity and detection limit is still required to permit the use of the BSA–GLA/BSA–GLA–XOD–PNP bilayer biosensor for reliable phosphate determination in drinking and other natural waters. In spite of this, the biosensor was found to be stable for 21 days, achieving a reproducibility of 5.7% rsd ($n = 5$) for potentiometric measurements during this period. However, the potentiometric response of the bilayer biosensor decreased by 10% after 21 days, possibly due to the gradual leaching of enzyme into buffer solution or deactivation of the enzymes. Nevertheless, the stability of the BSA–GLA–XOD–PNP biosensor is still much better than those of other phosphate biosensors [3]. Male and Luong [10] reported that the use of the same bienzyme system on reactivated nylon membrane resulted in a loss of 30% of its response in three weeks. The stability of the BSA–GLA/BSA–GLA–XOD–PNP bilayer biosensor is also far superior than achieved with a PPy-based phosphate potentiometric biosensor which lost 50% of its initial sensitivity after only 8 days and stabilized at 20% of its initial sensitivity after 2 weeks [50]. Despite the decrease in potentiometric response of the phosphate bilayer biosensor observed in the present study, the resulting response is still useful for quantification of phosphate by standard addition methods. However, when the BSA–GLA/BSA–GLA–XOD–PNP bilayer biosensor is stored in a buffer solution, the enzyme stability is improved and optimum potentiometric response is obtained.

4. Conclusions

This study demonstrates that chemical cross-linking of XOD and PNP (1:8) with a mixture of BSA and GLA is effective for fabrication of a reliable and stable potentiometric BSA–GLA/BSA–GLA–XOD–PNP bilayer biosensor for phosphate detection. A minimum detectable concentration of 20 μM was achieved with the bilayer biosensor for phosphate with a linear concentration range of 40–100 μM, which was extended slightly to 40–120 μM with a logarithmic plot of concentration versus potential. Although, the minimum detectable concentration was not as good as that of a previously reported PPy-based phosphate potentiometric biosensors [50], the stability of the bilayer biosensor is far more superior and its linear concentration range is much broader. Also, the minimum detectable concentration of 20 μM achieved with the bilayer biosensor is as good as those reported by other researchers for phosphate amperometric biosensors [11,18,28,44]. Furthermore, the process of fabrication of the BSA–GLA/BSA–GLA–XOD–PNP bilayer biosensor was simpler and more direct than most previously reported methods. Table 1 summarises the optimum conditions for fabrication of the BSA–GLA/BSA–GLA–XOD–PNP bilayer biosensor. Further investigations are being conducted to improve the minimum detectable phosphate concentration and study the effects of interferents on the biosensor response.

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