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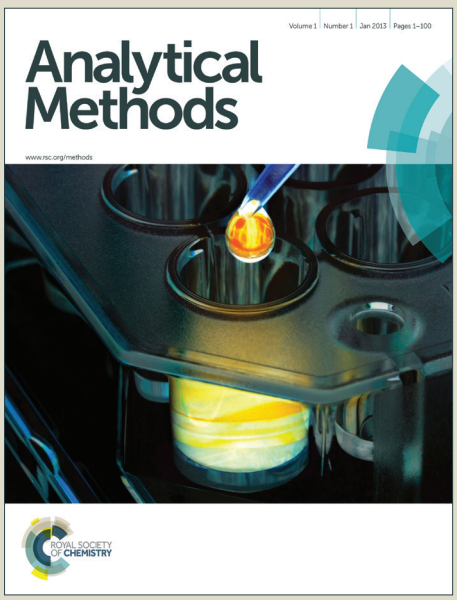

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A Potentiometric Phosphate Biosensor Based on Entrapment of Pyruvate Oxidase in Polypyrrole Film

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Abstract: A polypyrrole based potentiometric phosphate biosensor has been fabricated by entrapment of pyruvate oxidase in a polypyrrole film. The optimum condition for the fabrication of the biosensors were 0.3 M pyrrole, a polymerization time of 120 s, an applied current density of 0.05 mA/cm^2 and 2 U/mL of pyruvate oxidase. Under these conditions, the polypyrrole-pyruvate oxidase (PPy-PyOx) biosensor enabled the achievement of a minimum detectable concentration of 3 µM phosphate, a linear concentration range of 15-400 µM (r^2 = 0.980). The biosensor was successfully applied to the detection of phosphate in lake water samples with excellent recoveries ranging from 99–100%.

Key words: Potentiometry, pyruvate oxidase, galvanostatic polymerization, phosphate, entrapment.

1. Introduction

Phosphate is an important environmental and clinical analyte which can be present in an inorganic and/ or organic form [1-3]. Phosphates enter waterways from human and animal waste, phosphorus rich rock formation, laundry, cleaning, industrial effluents, and fertilizer runoff. High phosphate level is detrimental to the environment and can lead to eutrophication which disrupts aquatic life cycles [2-6]. Phosphate levels higher than 0.1 mg/L [7] may lead to many health concerns if it enters ground and surface water due to agricultural related activities [3, 8]. However, phosphate is also an essential component of nucleotide adenosine triphosphate (ATP) and nucleic acids (deoxyribonucleic acid and ribonucleic acid) [3]. High phosphate concentrations in the body fluid have been associated with the diagnosis of hyperparathyroidism, vitamin D deficiency, hypertension, mineral and bone disorder and fanaconi [3, 9]. There is therefore a need for a sensitive, efficient and reliable measurement method to determine low concentration of the phosphate ion in water and environmental samples. Various analytical methods have been developed to measure phosphate in environmental, industrial, biological and clinical samples [10-18].

Conventional analytical methods commonly used to measure phosphate are chromatographic [19-22] and spectrophotometric [7, 16, 23, 24] techniques. However, these methods are time consuming, laborious, require expensive instrumentation, require well trained operators, and often employ potentially carcinogenic chemicals for the analysis [2]. A simple and rapid alternative is the use of a biosensor which has considerable advantages over other conventional methods, including high selectivity, simplicity of use and possibility for miniaturization.

Various enzymatic biosensors for the detection of phosphate have also been developed [25-31] and have been extensively reviewed [1, 4, 12, 17]. The construction of phosphate biosensors have, in particular, been based on mono- or multi-enzymatic reactions where phosphate acts as an inhibitor or substrate. Enzymes such as purine nucleoside phosphorylase (PNP) and xanthine oxidase (XOD), alkaline phosphatase and glucose oxidase, phosphorylase A, phosphoglucomutase and glucose-6-phosphate dehydrogenase, and pyruvate oxidase (PyOx) have been used for fabrication of phosphate biosensors [9, 26-29, 32-37]. However, some of these methods have poor reproducibility, unstable response, poor enzyme activity, slow response time and poor spatially controlled deposition of enzyme [9]. The cost of the required enzyme(s) is also an important consideration. In addition, the use of a mono-enzyme involves a simple procedure for enzyme immobilisation and reduces interferences from sample constituent [2]. Furthermore, based on previous studies, comparable sensitivity and detection limit achieved with multi-enzyme systems [27,32,38] can also be obtained with single enzyme systems [9,37]. For example, a detection limit of 0.1 µM phosphate was obtained with a multi-enzyme system which employed PNP and XOD as enzymes [38], yet the same detection limit of 0.1 µM was also successfully achieved with a single enzyme system which employed PyOx [37]. This example clearly indicates that PyOx satisfies all the requirements for achieving a more cost effective enzyme system for phosphate detection and is therefore chosen as the enzyme to be employed in the present study. However, most of the previous studies on the fabrication of phosphate biosensors with PyOx use large quantities of the enzyme [2, 31, 39]. For example, 1200 U/mL of PyOx was immobilized on the surface of a screen printed platinum working electrode with nafion for the detection of phosphate [2], while in another study [39] 700 U/mL of PyOx was immobilized on aminoalkylated controlled pore glass by crosslinking with glutaraldehyde for phosphate detection. Although these are based on use of a mono-enzymatic reaction, the use of large quantities of PyOx is not economical and can limit the wider use of these biosensors. PyOx in the presences of some co-factors such as flavin adenine dinucleotide (FAD) and thiamine pyrophosphate (TPP) catalyses the oxidative decarboxylation of pyruvate to yield acetylphosphate, CO_2 and H_2O_2 as follows:

$$\text{Pyruvate} + \text{Phosphate} + O_2 \xrightarrow{\text{PyOx}} \text{Acetylphosphate} + CO_2 + H_2O_2$$

The electroxidation of H_2O_2 results in the generation of hydrogen ions, as shown below. This consequently decreases the pH of the solution, causing a change in the potential that is proportional to the phosphate concentration:

$$H_2O_2 \longrightarrow O_2 + 2H^+ + 2e^-$$

This study aims at developing a strategy for lowering the PyOx concentration used in the fabrication of phosphate biosensors, while still achieving good sensitivity and selectivity. In particular, we aim at achieving this by employing a polypyrrole (PPy) film because of its excellent properties, such as biocompatibility and good electrical properties [40]. Galvanostatic polymerisation, which will be employed

for this purpose, is particularly amenable to the localization of enzymes to small or defined electrode geometries [41]. To our knowledge, there is no previously reported study based on the entrapment of PyOx in PPy films for the development of a potentiometric phosphate biosensor. Furthermore, most of the reported PyOx-based phosphate biosensors are based on the amperometric measurement of H_2O_2 liberated by the enzymatic reaction for phosphate detection [2, 8, 9, 37, 42].

Important considerations in this study for achieving optimum detection of phosphate with the resulting PPy-PyOx biosensor will include the influence of pyrrole (Py) concentration, PyOx concentration, applied current density, polymerization time, FAD concentration, TPP concentration and pyruvic acid concentration. Also, the influence of potential interferants, such as SO_4^{2-}, Cl^-, and NO_3^- on the sensitivity of the PPy-PyOx biosensors will be investigated. Furthermore, the application of the PPy-PyOx biosensor to the reliable determination of phosphate in lake water will be investigated.

2. Experimental

2.1. Chemicals and reagents

PyOx (EC. 1.2.3.3 67 units/mg) from *Aerococcus sp*, Py, pyruvic acid, FAD, TPP, and potassium phosphate, were all purchased from Sigma-Aldrich (Australia). Magnesium chloride-6-hydrate ($MgCl_2 \cdot 6H_2O$), citric acid and sodium citrate were purchased from Rowe Scientific Pty Ltd Australia. Pyrrole was distilled under vacuum at 130 ^oC prior to use and this was stored in an aluminium foil-covered sample bottle in a freezer to prevent UV degradation until required for use. All chemicals used were of analytical grade and used as received unless otherwise stated. Citrate buffer of 0.01 mM (pH 7) which contained 70 µM TPP, 10 µM FAD, 3 mM $MgCl_2 \cdot 6H_2O$ and 0.5 mM pyruvic acid, was used for the potentiometric measurements. All solutions were prepared with Milli-Q water (18.2 MOhm cm).

2.2. Instrumentation

A potentiostat/galvanostat was used during the fabrication of PPy-PyOx biosensor, as well as for potentiometric measurements. Potentiometric measurements were performed in 0.01 mM citrate buffer of pH 7 containing 70 µM TPP, 10 µM FAD and 3 mM $MgCl_2 \cdot 6H_2O$. Cyclic voltammetry was performed by scanning from +600 mV to −1000 mV with a Voltalab 40 PGZ 301 voltammeter and recorded on a P-4 (2.8 GHz) computer using the VoltaMaster 4 version 2.00 (Radiometer Copenhagen). Platinum working electrode (0.0079 cm^2 surface area), platinum wire auxiliary electrode and an Ag/AgCl reference electrode were purchased from EDAQ Pty Ltd (NSW, Australia). A Sybron Thermolyne stirrer model S – 17410 was used for stirring during potentiometric measurements.

2.3. Electrode preparation

The platinum working electrode surface was polished using alumina powder < 50 µm on a polishing pad and rinsed with Milli-Q water, methanol and once again with Milli-Q water and then polished with 15, 3 and 1 µm diamond polish, rinsed with methanol and Milli-Q water. The working electrode

was sonicated for 600 s in a mixture of Milli-Q water and methanol to remove any alumina particles and finally dried in vacuum. Electrochemical cleaning of the working electrode was accomplished in a 1.0 M H_2SO_4 solution by cycling the electrode potential between -200 and +1450 mV versus Ag/AgCl for approximately 600 s at a sweep rate of 75 mV/s, until a constant current-voltage feature was obtained.

Electropolymerization of Py on the working electrode was carried out galvanostatically in an unstirred monomer solution. This approach was based on galvanostatic entrapment of PyOx in a PPy film in the presence of a supporting electrolyte (potassium chloride). The monomer solution contained 0.3 M Py, 2 U/mL PyOx, 0.15 M potassium chloride (unless otherwise stated). All solutions were purged with nitrogen gas for 180 s to remove dissolved oxygen prior to electropolymerisation. After the galvanostatic film formation, the working electrode was rinsed several times under a stream of Milli-Q water to remove any loosely bound PyOx and monomer prior to potentiometric measurement.

2.4. Potentiometric Measurements

A two-electrode cell, which consisted of a PPy-PyOx working electrode and an Ag/AgCl reference electrode, was used for the potentiometric measurements at equilibrium. In our work, 2 mL of 0.01 mM (pH 7) citrate buffer (containing 0.5 mM pyruvic acid, 10 μM FAD, 3 mM $MgCl_2 \cdot 6H_2O$, and 70 μM TPP), and was stirred continuously during the measurement. In order to measure the potentiometric response of the biosensors, a standard phosphate solution was injected periodically after a steady-state potential between the sensor and the reference electrode has been achieved. The resulting potential change was then measured and recorded for varying phosphate concentrations. All experiments were carried out in triplicate at room temperature.

3. Results and discussion

3.1 Enzyme immobilization and characterization

The entrapment of PyOx in the PPy film was confirmed by comparing the chronopotentiograms (potential-time curves) obtained during polymerization of Py monomer in presence of Cl^- ions and PPy-PyOx, respectively. Fig. 1(A) illustrates that the lowest initiation potential of 584 mV was obtained for PPy-Cl^- film compared to the 694 mV for PPy-PyOx. Evidently, the larger size of the enzyme, compared to the chloride ion, makes the polymerization more difficult [43]. The higher stabilization potential obtained for the formation of the PPy-PyOx film is also indicative of the high resistance and lower conductivity of the PPy-PyOx solution.

The electrochemical behaviour of PPy-PyOx film was investigated in 0.1 M citrate buffer by cyclic voltammetry between -800 and +600 mV at a scan rate of 100 mV/s. As can be seen in Fig. 1(B), the shapes of the voltammograms changed with the inclusion of PyOx in the PPy film, compared to PPy-Cl^- voltammogram. The characteristic anodic and cathodic redox peaks for PPy occurred at -106 mV and -400 mV. Both peaks were considerably suppressed with the incorporation of PyOx which renders the PPy film less conductive, confirming PyOx entrapment in the film.

Fig. 1

3.2 Optimization of polymerization conditions

3.2.1 Choice of pyrrole concentration

Entrapment of PyOx in the PPy film on the surface of electrode can be influenced by Py concentration used. Fig. 2a shows that the use of increasing Py concentration for the formation of PPy-PyOx film resulted in increasing phosphate potentiometric response. However, beyond 0.3 M, a reduction in phosphate response was observed. This is due to the increased PPy-PyOx film thickness which, in turn, increased the diffusion barrier and resulted in a reduction in the phosphate response. The increased diffusion barrier limits the ability of the catalytic product (H_2O_2) to reach the electrode surface [43]. Consequently, 0.3 M Py was chosen as optimum for the film formation for all subsequent measurements. This is lower than the 0.5 M Py used for co-immobilization of PNP and XOD for phosphate detection [44]. The higher concentration of Py (0.5 M) required in the other study for the co-entrapment of PNP and XOD may be due to the large molecular size and quantity of PNP and XOD that was entrapped, compared to that used in the present study.

3.2.2 Influence of current density and polymerization time

The phosphate potentiometric response increased slightly with increasing applied current density up to 0.05 mA/cm^2 as shown in Fig. 2b. Further increase in the current density leads to a decrease in the potentiometric phosphate response. This is again due to increased film thickness and associated effect of increased diffusion barriers [43]. The use of higher current density may also affect entrapment of PyOx due to rapid PPy film formation, which is accompanied by an increased diffusion barrier. Therefore, the PPy-PyOx film formed for the rest of the study was achieved with a current density of 0.05 mA/cm^2. A previous study used an applied current density of 0.25 mA/cm^2 to fabricate a PPy-based bilayer biosensor for potentiometric determination of phosphate [28], which is higher than the optimum in this present study and may be due the increased thickness of PPy bilayer film used in that study [45].

The polymerization time is critical in film formation, and also for enzyme loading in PPy film. As shown in Fig. 2c, increase in the polymerisation time results in increasing potentiometric phosphate response. Increasing the polymerisation time beyond 120 s led to a sharp decrease in the potentiometric phosphate response. This is also due to increase in the film thickness and the associated effect of the increased diffusion barrier. Hence, a polymerization time of 120 s was adopted for formation of PPy-PyOx films for all other potentiometric measurements.

Fig. 2

3.2.3 Effect of electrolyte concentration

The presence of KCl in the monomer solution is critical for the formation of the PPy-PyOx film, because KCl serves as a supporting electrolyte. However, the concentration of KCl used must be controlled to ensure that the formation of PPy-Cl film does not predominate and the formation of this film is likely to result in increased film thickness. In contrast, the absence of KCl may lead to higher initiation potential, which may also lead to denaturation of the enzyme and low conductivity of the enzyme solution [43]. Fig. 3a shows that, with the addition of KCl up to 0.15 M to the monomer solution, the phosphate potentiometric response increased. However, beyond 0.15 M, a gradual reduction of the potentiometric phosphate response was observed due to increased polymer film formation, and the associated increase in the diffusion barrier. Consequently, a KCl concentration of 0.15 M was used in all subsequent work.

Fig. 3

3.2.4 Optimization of enzyme

Fig. 3b shows that the potentiometric response for phosphate increased rapidly with increasing PyOx concentration used for growth of the PPy-PyOx film. This confirms that the PyOx was successfully entrapped within the PPy film, as a result of which the film thickness and polymer permeability controlled the sensitivity of the PPy-PyOx film to phosphate. As shown in Fig. 3b, an optimum response was obtained with the incorporation of 2 U/mL of pyruvate oxidase. Beyond this enzyme concentration, the amount of entrapped PyOx increased with corresponding increase in the film thickness and associated diffusion barrier. Consequently, a steady decrease in the phosphate response was observed with increasing PyOx concentration from 2 U/mL to 6 U/mL. The optimum PyOx concentration in this study is much lower than those reported in several previous studies which ranged from 20-1200 U/mL [2, 31, 34, 46-49]. However, it is closer to the optimum concentrations of 2.5 U/mL [50] used in an amperometric phosphate biosensor based on the immobilization of PyOx with gelatin (4.76 mg/mL) by cross-linking with glutaraldehyde (2.5%). The low optimum PyOx concentration used in this study may be due to the excellent conductive properties of PPy, ability of PPy to maintain the accessibility of the catalytic site due to the permeability of the film to the analyte, and also the entrapment of PyOx in the matrices of PPy film prevents the enzyme from being leached out from the surface of the electrode [51], hence maintaining the sensitivity and stability of the biosensor.

3.3 Optimization of measurement parameter

3.3.1 Effect of pH, buffer concentration and pyruvic acid

One of the parameters that can influence the potentiometric response at the PPy-PyOx electrode is the pH of the measurement solution. Fig. 4 (a) shows that phosphate response increased with increasing pH up to 7. Beyond pH 7, the phosphate response decreased. This is due to reduced enzyme activity at the higher and lower pHs [43]. This optimum pH of 7 is in agreement with those reported previously for

the optimum performance of a phosphate biosensor based on immobilization of PyOx in a nafion matrix covered with a poly (carbomoyl) sulphonate hydrogel on a screen printed electrode [2]. Fig. 4 (b) shows the influence of buffer concentration on the phosphate response. An increase of the buffer concentration beyond 0.01 mM decreased the phosphate response. Consequently, 0.01 mM buffer solution was used for further investigations. Another study [2] which used a screen printed electrode for fabrication of a PyOx biosensor used 0.1 M citrate buffer for the amperometric measurement of phosphate. Unlike for amperometric biosensors, higher buffering capacity resulting from buffer concentration will lead to a decrease in potentiometric phosphate response [5, 29]. 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 the lower potentiometric response for phosphate. Also the observed lower phosphate sensitivity may be due to the lowering of the phosphate activity under conditions of high ionic strength. Similar reduction in potentiometric response for phosphate at higher buffer concentration has been previously reported [29] in a study where PNP and XOD were cross-linked with glutaraldehyde and bovine serum albumin for potentiometric detection of phosphate. An optimum concentration of 0.05 M Tris-HCl buffer (pH 6.8) was used in that study and a further increase in the buffer concentration led to a sharp decrease in the potentiometric phosphate response.

Fig. 4

3.3.2 Influence of Co-factors

The presence of FAD, TPP, magnesium chloride and pyruvic acid is essential for the catalytic activity of PyOx [46]. PyOx is a homo-tetrameric enzyme and each subunit (62 kDa) binds to FAD noncovalently and also binds to TPP loosely, while Mg^{2+} is the metal cation for the reaction which is catalysed by TPP [46]. For this reason, the optimum concentrations of these co-factors were also investigated. Pyruvic acid is critical in the determination of phosphate concentration because it is a co-substrate in the enzymatic reaction [47]. The potentiometric response for phosphate, as shown in Fig. 5, increased with increase in pyruvic acid concentration. The optimum pyruvic acid concentration was 0.5 mM and further increase in concentration led to a steady decrease in the phosphate potentiometric response. Hence, a pyruvic acid concentration of 0.5 mM was adopted for all other measurements. In other studies, 1 mM pyruvic acid concentration was used [2, 9, 49]. The low pyruvic acid concentration used in this study is due to the considerably less enzyme employed with the PPy-PyOx biosensor. Similarly, to determine the optimum TPP concentration, its concentration was varied from 30 to 90 µM. An increase in the phosphate potentiometric response was observed with increase in the TPP concentration, as shown in Fig. 5 (b). The optimum potentiometric response for phosphate was observed in the presence of 70 µM TPP. Further increase in TPP concentration led to a decrease in the potentiometric phosphate response. This may be due the fact that the required maximum TPP

concentration for the decarboxylation of pyruvate by PyOx was already exceeded [34, 52, 53]. Hence, 70 µM of TPP was used in all subsequent experiments.

Fig. 5

FAD concentration was varied from 5 µM to 20 µM as shown in Fig. 5 (d), to determine its influence on phosphate response. The potentiometric phosphate response increased with FAD concentration. Increasing the FAD concentration beyond 10 µM decreased the potentiometric phosphate response. Similar to TPP, FAD is also required for the decarboxylation of pyruvate by PyOx, but has very little effect on the enzymatic activity, probably due to its high energy bond with PyOx [53]. Hence, 10 µM FAD was employed for further studies. As seen in Fig. 5 (c), the concentration of magnesium chloride was varied from 1 mM to 4 mM. The optimum potentiometric phosphate response was observed in the presence of 3 mM magnesium chloride concentration. Hence, 3 mM magnesium chloride concentration was used for the rest of the studies. In another study [42], 5 mM $MgCl_2$, 0.6 mM TPP, 10 µM FAD and 1 mM pyruvic acid was used in the measurement solution. Mg^{2+} is required for the binding of TPP to PyOx in the enzymatic decarboxylation of pyruvate [54], the higher concentration of Mg^{2+} that was used in the other study [42] may be due to the use of high PyOx concentration in that study

3.4 Analytical performance

Fig. 6a shows the potentiometric response obtained with the PPy-PyOx biosensors for increasing addition of phosphate to the measurement solution. The potential of the biosensors increased with increasing addition of phosphate standard. Fig. 6b shows the plot of potential change versus -log [PO_4^{3-}], which demonstrated Nernstian behaviour and linear concentration range of 15-400 µM (r^2 = 0.980, N=18), (n= 3) and a slope (sensitivity) of 14.97 mV/decade. Fig. 6c shows the linear portion of the calibration plot ranging from 15 - 250 µM, which compares with a range of 20 - 200 µM, previously reported [28]. Based on a signal to noise ratio of 3, a detection limit of 3 µM phosphate was estimated. This is far superior than those obtained with the bienzyme system, based on PNP and XOD, for phosphate detected [5, 28, 44, 45].

Fig. 6

3.5 Interference studies

The selectivity of the PPy-PyOx biosensor to phosphate ions was evaluated in the presence of other anions such as Cl^-, SO_4^{2-}, and NO_3^-. The response for 50 µM of phosphate was not affected with the addition of chloride, sulfate, and nitrate up to 200, 300 and 150 µM respectively, in the measurement solution. There was a decrease in the potentiometric phosphate response at higher concentrations of

Cl^-, SO_4^{2-}, and NO_3^-. However, the concentrations of these anions in rivers and lake water are much lower and, hence, less likely to interfere with phosphate analysis using the PPy-PyOx biosensor.

3.6 Application of PPy-PyOx biosensor to water samples

The PPy-PyOx biosensor was used to determine phosphate concentration in three separate lake water samples collected from the Gippsland lakes (Victoria, Australia). Recovery studies for the lake water with the PPy-PyOx potentiometric biosensor at different phosphate concentrations revealed in Table 1 that excellent recoveries in the range of 99 -100 % with a R.S.D of 0.06 - 0.16 (n = 3) were achieved. The concentration of phosphate in the lake water sample was 0.08 ± 0.02 µM, which is within the range of the values obtained for studies conducted on 109 lakes in another study (0.02-1.47 µM) [55].

Table 1

3.7 Conclusion

PPy-PyOx biosensors have been successfully fabricated for the potentiometric determination of phosphate. A linear concentration range of 15 - 400 µM and a detection limit of 3 µM was achieved with PPy-PyOx biosensors. The concentration of PyOx (2 U/mL) used in the fabrication of PPy-PyOx biosensor in this study is greatly reduced compared to those used in other studies. The PPy-PyOx biosensor has been successfully used for the determination of phosphate in lake water samples. Excellent recovery of 99-100% phosphate in lake water was achieved with the biosensor. The sensitivity achieved for the potentiometric detection of phosphate (15 mV/decade) is low and further investigation is needed to improve this. It may be possible to achieve some improvement by incorporation of nanomaterials, such as gold nanoparticles and carbon nanotubes, to provide larger surface area and improve sensitivity.

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Table 1. Phosphate recovery in lake water sample.

Concentration added, μM	Concentration found, μM	Recovery %
0.00	0.08 ± 0.02	-
5.00	5.04 ± 0.01	99.00 ± 0.16
10.00	10.05 ± 0.01	100.00 ± 0.08
50.00	50.18 ± 0.02	100.00 ± 0.06

Figure captions

Fig. 1 Electrochemical formation and characterization of PPy films. (A) Chronopotentiogram of (a) PPy-Cl and (b) PPy-PyOx. (B) Cyclic voltammograms obtained for (a) PPy-Cl and (b) PPy-PyOx. Scan rate of 100 mV/s. Film growth conditions: (a) 0.3 M [Py], 0.15 M KCl (b) 0.3 M [Py], 0.15 M KCl, 2 U/mL of PyOx at a current density of 0.05 mA/cm^2 and polymerization time of 120 s.

Fig. 2 Influence of (a) Py concentration (b) current density and (c) polymerization time used for the formation of PPy-PyOx film on potentiometric phosphate response. PyOx= 2 U.mL^{-1}, phosphate concentration was 50 µM. Other film formation conditions were same as in Fig. 1. Measurement solution was 0.01 mM (pH 7) citrate buffer which contained 0.5 mM pyruvic acid, 10 µM FAD, 3 mM $MgCl_2 \cdot 6H_2O$, and 70 µM TPP. Potentiometric response is measured as potential change which is the difference between the final potential (in presence of phosphate) and the initial (background) potential (in absence of phosphate).

Fig. 3 Influence of (a) KCl, (b) PyOx concentration used for the formation of PPy-PyOx film on potentiomentric phosphate response. PyOx= 2 U.mL^{-1}, phosphate concentration was 50 µM. Other film formation conditions were same as in Fig. 1 and measurement solution was as given in Fig. 2.

Fig. 4 Influence of (a) pH and (b) citrate buffer concentration on the potentiometric response obtained with the PPy-PyOx biosensor. PyOx= 2 U.mL^{-1}, phosphate concentration was 50 µM. Other film formation conditions were same as in Fig. 1 and measurement solution was as given in Fig. 2.

Fig. 5 Optimization of (a) pyruvic acid, (b) TPP, (c) $MgCl_2$, and (d) FAD for potentiometric detection of phosphate. Film formation conditions were same as in Fig. 1 and measurement solution was as given in Fig. 2.

Fig. 6 Typical potentiometric responses (a) obtained for phosphate with the PPy-PyOx biosensor and (b) calibration curve. Successive addition of 50 µM phosphate was used and the inset (c) is the linear portion of the calibration curve. Film formation conditions were same as in Fig. 1 and the measurement solution contained 0.01 mM citrate buffer at pH 7 containing 70 µM TPP, 10 µM FAD, 3 mM $MgCl_2$ and 0.5 mM pyruvic acid.

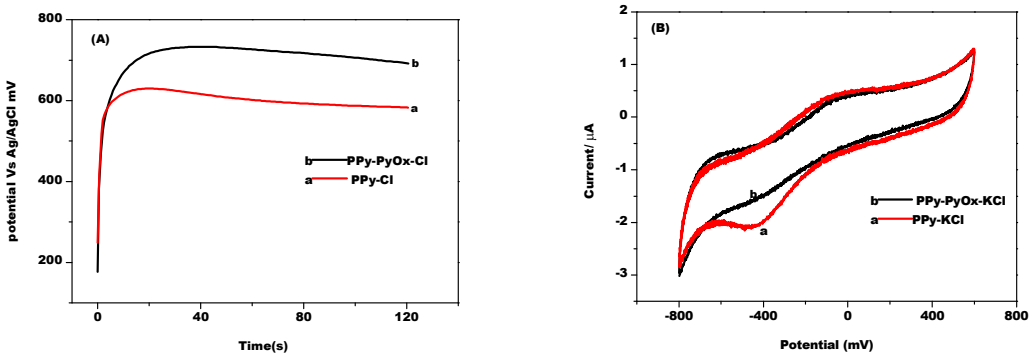

Fig. 1

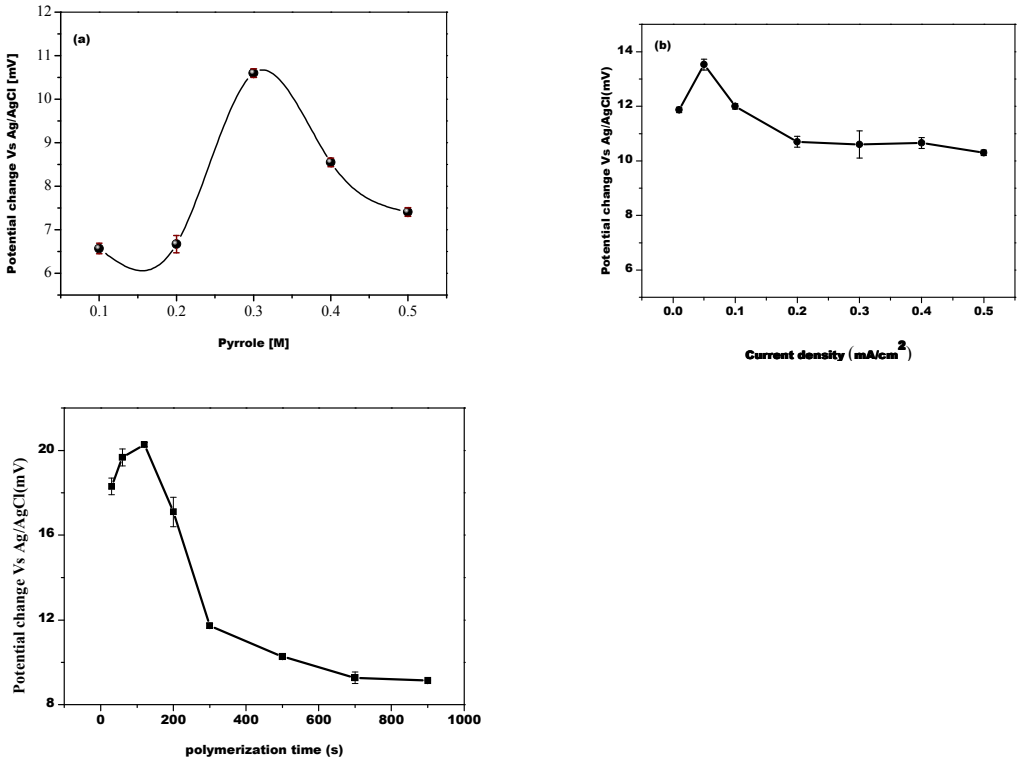

Fig. 2

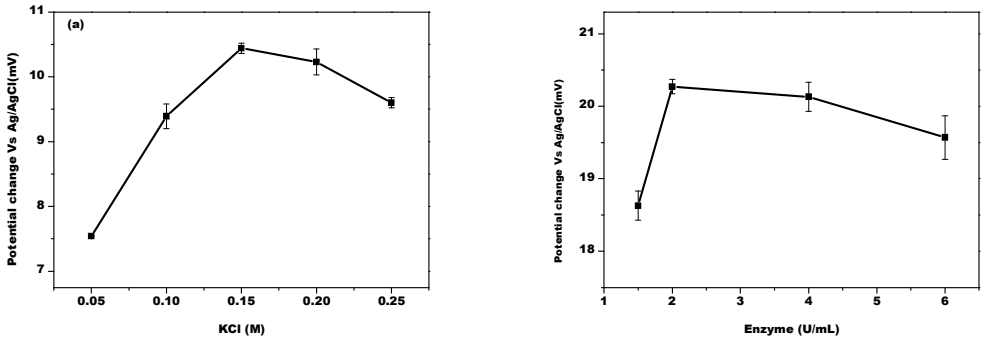

Fig. 3

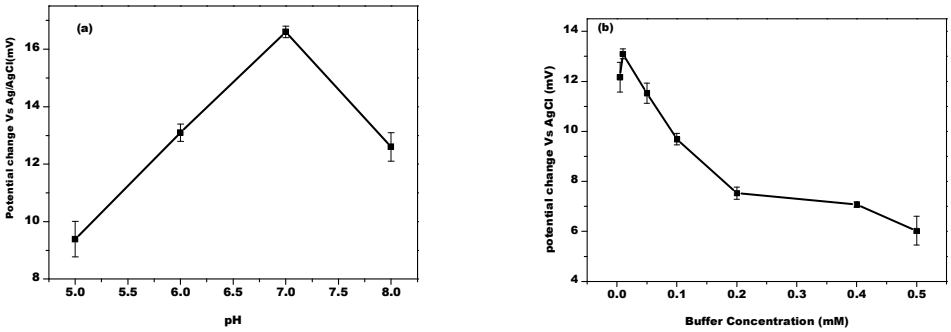

Fig. 4

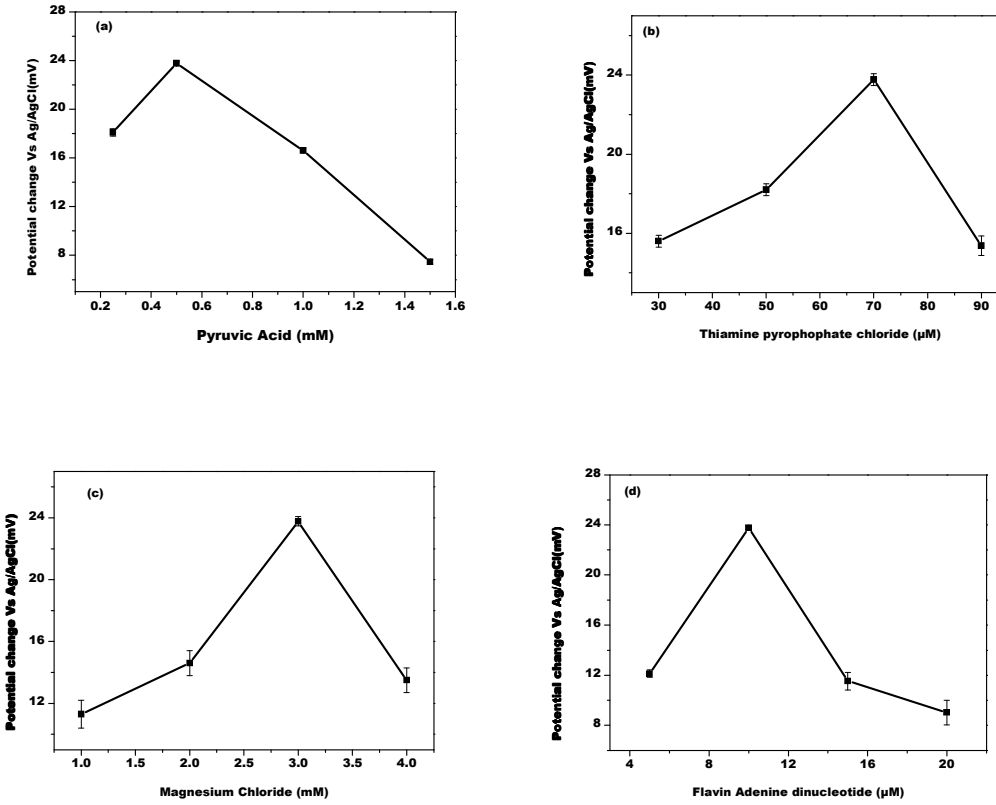

Fig. 5

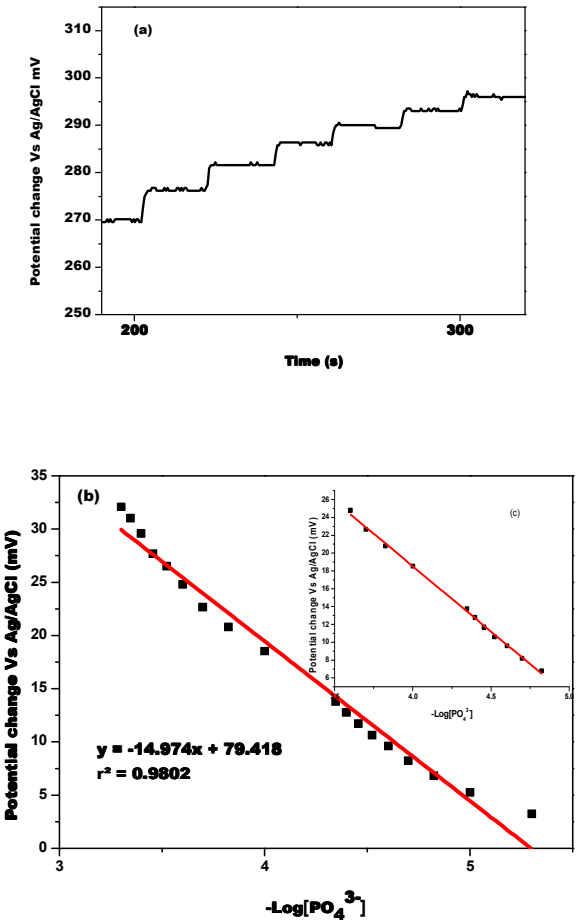

Fig. 6