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Alkaline phosphatase inhibition based conductometric biosensor for phosphate estimation in biological fluids

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ABSTRACT

Determination of phosphate ions concentration is very important from both, environmental and clinical point of view. In this study, a simple and novel conductometric biosensor for indirect determination of the phosphate ions in aqueous solution has been developed. The developed biosensor is based on the inhibition of immobilized alkaline phosphatase activity, in the presence of the phosphate ions. This is the first time we developed a mono-enzymatic biosensor for indirect estimation of phosphate ions. The developed biosensor showed a broad linear response (as compared to other reported biosensors) for phosphate ions in the range of 0.5–5.0 mM (correlation coefficient=0.995), with a detection limit of 50 μM. Different optimized parameters were obtained as the buffer concentration of 30 mM, substrate concentration of 1.0 mM, and a pH of 9.0. All the optimized parameters were analyzed by analysis of variance, and were found to be statistically significant at a level of $\alpha=0.05$. The developed biosensor is also suitable to determine the serum phosphate concentration, with a recovery of 86–104%, while a recovery of 102% was obtained from the water samples that were spiked with 500 μM phosphate. A relative standard deviation in the conductance response for five successive measurements ($n=5$) did not exceed 7%, with a shelf life of 30 days. With a lower detection limit and a higher recovery, the biosensor provides a facile approach for phosphate estimation in biological fluids.

1. Introduction

Phosphorus is the sixth most abundant element in the human body. It is critical for bone mineralization, cellular structure, genetic coding, and energy metabolism. The normal range of phosphate in an adult human serum is 1.1–1.4 mM (Bansal, 1990), which is determined by the dietary intake, gastrointestinal absorption, urinary excretion, and shifts between the intracellular and extracellular spaces. Abnormalities in any of these steps can results either in hypophosphatemia (lower serum phosphate level) or hyperphosphatemia (higher serum phosphate level) (Popovtzer and Levi, 2001). Diagnosis of these two conditions in early stages is a prerequisite for their effective treatment, as a prolonged persistence of the altered phosphate levels has major consequences. An increment in the phosphate concentration, i.e. hyperphosphatemia cause hypocalcemia by precipitating calcium, decreasing vitamin D production, and interfering with parathyroid hormone-mediated bone resorption (Walsdorf and Alexandrides, 2005). On the other side, a decreased phosphate level results into decreased erythrocyte 2,3 di-phosphoglycerate levels, which increases the hemoglobin's affinity for oxygen, and consequently reduces the oxygen release at tissue level (Popovtzer and Levi, 2001). Current method which is widely used for the determination of phosphate ions includes a spectrophotometric method in which the inorganic phosphorus reacts with the ammonium molybdate in acidic conditions to produce phosphomolybdate complex according to the following reaction:

Phosphate + ammonium molybdate

$$\xrightarrow{H_2SO_4}$$ *Phosphomolybdic complex* (*Unreduced*)

This phosphomolybdic complex absorbs light at 340 nm, and an increase in absorbance is then correlated to the concentration of inorganic phosphate present in the sample. However, these standard methods for phosphate determination are difficult to be incorporated for *in situ* analysis and thus, incur significant cost for large scale determination. Moreover, use of some carcinogenic chemicals such as molybdenum make this procedure health hazardous (Gilbert et al., 2011; Zhang et al., 2008). In addition to this, phosphate sensors in the form of ion selective electrodes have been developed, but these ion selective membranes have a very poor selectivity for the phosphate due to its very high hydration energy (Sudduth et al., 2014). Also, the free energy of phosphate species is very small, and the large size of phosphate ions prohibits the use of size-exclusion principles for increased selectivity.

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Development of a biosensor is an alternative and effective analytical approach with minimum sample and time requirement. Few biosensor for phosphate estimation have been reported in literature (Cosnier et al., 1998; Guilbault and Nanjo, 1975; Mousty et al., 2001; Su and Mascini, 1995), but most of them were multi-enzymatic, which have disadvantages of being complicated and expensive.

This paper describes the development of a novel conductometric biosensor using immobilized alkaline phosphatase, for indirect determination of phosphate ions in the aqueous solutions. Alkaline phosphatase, a nonspecific phosphomonoesterase, is reported to be competitively inhibited by the phosphate (Coburn et al., 1998; Fernley and Walker, 1967; Torriani, 1968), but it has never been reported to be used solely in the biosensor development for phosphate estimation. However, few works used alkaline phosphatase for the analysis of heavy metals and pesticides (Bachan Upadhyay and Verma, 2013; Berezhetskyy et al., 2008; García Sánchez et al., 2003; Mazzei et al., 2004; Prieto-Simón et al., 2006). The enzyme was immobilized on a glass surface with the help of cysteine functionalized silver nanoparticles, and further incorporated with a conductivity transducer to determine the inhibitory effect of phosphate on the immobilized enzyme. The developed biosensor was finally used for its potential application in determining the phosphate level of serum and tap water.

2. Materials and methods

2.1. Chemicals

Potassium chloride and sodium monobasic phosphate were purchased from Himedia (India), while p-nitrophenyl phosphate (99% purity) was obtained from Loba Chemie. All other reagents were of analytical grade, and were used without any further treatment. Deionized (DI) water from Millipore was used for the reagent preparation and throughout the process.

2.2. Preparation of enzyme immobilized glass tube

The enzyme, alkaline phosphatase (APase), was immobilized on the internal surface of a glass test tube by the method as reported (Upadhyay and Verma, 2014). Briefly, the glass test tube was silanized with (3-Mercaptopropyl) trimethoxysilane, and further treated with cysteine functionalized silver nanoparticles to generate a cysteine rich surface. The enzyme, alkaline phosphatase (1.2 U/mg), was then immobilized on the cysteine group of nanoparticles, by carbodiimide and glutaraldehyde activation of carboxyl and amino group respectively.

2.3. Biosensor design

Electrochemical experiments were performed with a conductometer (model LI-CON01, Labard Instruments, India), equipped with a temperature probe and a conductivity electrode. The electrode consists of two pairs of platinum electrodes separated by a distance of 1 cm. This electrode was kept in the center of the enzyme immobilized glass tube, along with the temperature probe. A schematic representation of enzymatic biosensor is presented in Fig. 1a. All the data were finally stored in a data recorder for interpretation.

2.4. Measurements

For biosensor, measurements were carried out in the enzyme coated glass tube containing Tris HCl buffer (30 mM, pH 9.0) and p-nitrophenyl phosphate substrate (1.0 mM, pH 9.0), with a final

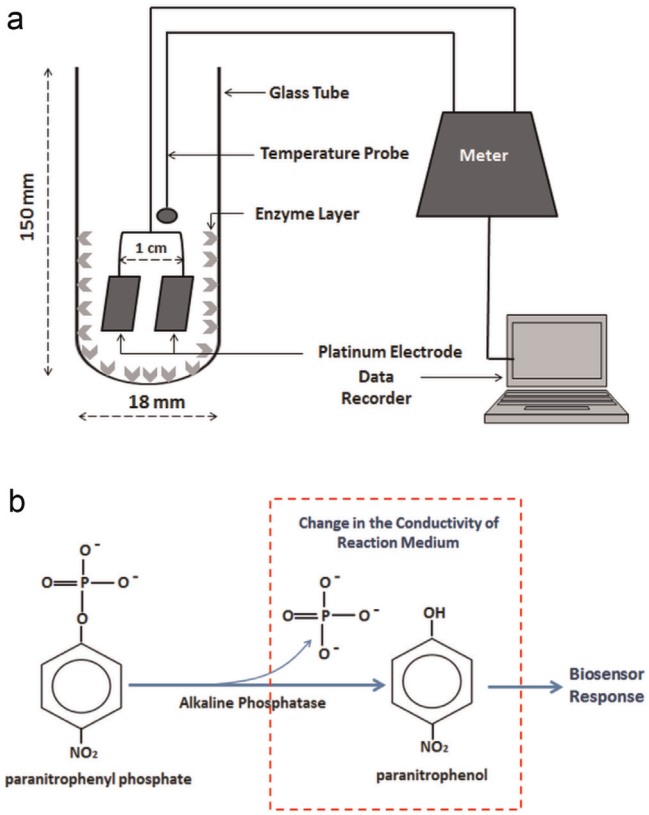

Fig. 1. Schematic representation of alkaline phosphatase based conductometric biosensor (a), and the enzymatic reaction for conductivity detection principle (b), for the determination of phosphate ions in aqueous solutions (a). The proposed biosensor consist of a platinum electrode, temperature probe, enzyme immobilized glass tube, conductivity meter and a data recorder.

reaction volume of 4 mL. All the measurements were carried out at 37 °C, and the sensor was calibrated with 0.1 N KCl before each measurement. To determine the linear response of biosensor, varying concentrations of phosphate were prepared in DI water. Prior to analysis, equal volumes (1 mL) of substrate and phosphate were mixed and then added to the enzyme immobilized glass tube. For blank samples (without inhibitor), DI water (1 mL) was used instead of phosphate, and added to substrate-buffer solution in the enzyme immobilized glass tube. After an incubation of 10 min at 37 °C, conductivity was again measured at the same temperature. Presence of phosphate ions in the sample interferes with the activity of immobilized enzyme, and results in the reduction of biosensor sensitivity to the substrate. The biosensor response was then measured by determining the change in the conductivity, and the percentage inhibition was calculated as

$$\textit{Percentage Inhibition}\ (\%) = 100 \times \left\{1 - \frac{dC_I}{dC_O}\right\}$$

Where,

dC_I is the change in conductivity in the presence of inhibitor, and

dC_O is the change in conductivity in the absence of inhibitor.

2.5. Reproducibility and storage stability

The reproducibility of the conductometric biosensor was also investigated by five successive measurements using the same biosensor, for a known phosphate concentration (3 mM). After each experiment, the biosensor was washed with 30 mM buffer (Tris HCl, pH 9.0) three times, and stored at 4 °C. For short term

and long term storage, the biosensor was kept in buffer filled condition (Tris HCl, 30 mM), and stored at 4 °C.

2.6. Statistical analysis

Optimization of parameters, such as buffer concentration, substrate concentration and pH were carried out in triplicate ($n=3$), and the results were statistically analyzed by analysis of variance (ANOVA). A mean, with standard deviation was calculated and reported using Origin Pro (Version 8).

3. Results and discussion

3.1. Alkaline phosphatase biosensor

The alkaline phosphatase conductometric biosensor is based on the change in the conductivity of reaction medium as a result of enzymatically conversion of the substrate, paranitrophenyl phosphate, to the product paranitrophenol according to the reaction as shown in Fig. 1b. The resultant conductivity change is then determined by a conductometric transducer, attached to a computer. The phosphate, a competitive inhibitor of alkaline phosphatase, competes with the substrate for the enzyme active site, and results in the interference of enzyme for substrate specificity.

3.2. Parameter optimization

3.2.1. Substrate concentration

Optimal parameters for APase conductometric biosensor were determined through the acquisition of a calibration curve, in the presence and absence of inhibitor. In the first step of this work, substrate concentration was optimized by varying it from 0.05 mM to 6 mM, and the biosensor response was analyzed. Fig. 2a shows the effect of substrate concentration on biosensor response, when exposed to 3 mM phosphate concentration. The enzyme activity follows a classical Michaelis–Menten behavior in the absence of inhibitor up to a substrate concentration of 6 mM. On further increasing the substrate concentration substrate concentration, all the enzymes become saturated with the substrate, and therefore no further changes in the biosensor signal was observed. However, in the presence of inhibitor and at a low substrate concentration (0.05–0.5 mM), a lower signal was observed ($p<0.05$), while on further increase in the substrate concentration, a continuous increment in the biosensor signal was observed up to 5 mM. However, the intensity of these signals was comparably lower than the earlier signals observed in the absence of inhibitor. At a higher substrate concentration of 6 mM or more, no effect of the inhibitor on the biosensor response was observed, and the biosensor signals were almost similar (as observed in the absence of inhibitor). These variations in the biosensor response may be due to the competitive nature of the inhibitor. At a lower substrate concentration, the inhibitor competes more effectively for the active sites of enzymes and thus, a lower activity in terms of biosensor signals was obtained. With increasing substrate concentration, the substrate begins to compete with the inhibitor, and an increment in the enzymatic activity was observed. At a very high concentration of 6 mM, the substrate totally out-competes the inhibitor and maximum enzyme activity was observed, similar to the activity obtained in the absence of inhibitor. To further confirm the competitive nature of the phosphate ions, spectrophotometric method was used to determine the initial rate of reaction, and a Lineweaver Burk plot was prepared. The plot further confirms the competitive nature of phosphate as an increment in the value of Michaelis constant (K_m) from 0.19 mM to 0.83 mM was obtained, with a similar V_{max} values of $\sim 67\ \mu M\ min^{-1}$ (Fig. 2b). At a concentration of 1 mM, sufficient stable signals with good sensitivity to the inhibitor was observed and thus, chosen as an optimum substrate concentration for biosensor response. Table a (supplementary data) shows the analysis of variance for the experimental data, and the results were found to be statistically significant at $\alpha=0.05$.

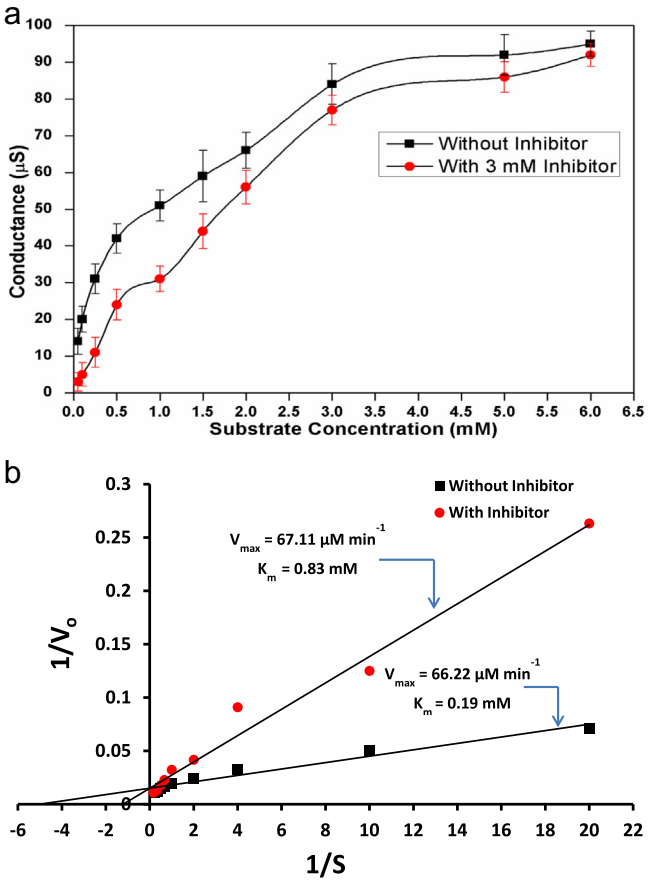

Fig. 2. Biosensor response at varying concentration of substrate in the presence and absence of inhibitor (a). All the measurements were conducted in Tris HCl buffer (30 mM, pH 9.0), and the biosensor shows better sensitivity for 3 mM inhibitor, at the substrate concentration of 1 mM. The competitive nature of the inhibitor was confirmed by Lineweaver–Burk plot (b).

3.2.2. Buffer concentration and pH

For conductometric measurements, the concentration of buffer is an important factor as a higher concentration will lead to a large fluctuationss in the biosensor response due to its high conductivity (Chouteau et al., 2004), while a lower buffer concentration may limit the enzyme activity. Fig. 3a shows the effect of different ionic concentration of buffer on the biosensor signals at 1 mM substrate concentration, when exposed to 3 mM phosphate. At lower buffer concentration (20 mM and 30 mM), a higher signal amplitude in terms of inhibitor sensitivity was observed, while on increasing the ionic concentration above 30 mM, a reduced response to the inhibitor was observed ($p<0.05$). The reduction in the biosensor response may be due to the high conductivity of the solution which creates a high background signals (Hanss and Rey, 1971; Lawrence and Moores, 1972). So, 30 mM was considered as the best ionic concentration for the biosensor response. Most of the conductometric biosensor has also been reported to be operated at a lower buffer concentration to avoid signal interferences (Dzyadevych et al., 2001; Wang et al., 2006; Zhylyak et al., 1995).

Since the enzymatic reaction also alters the pH of the reaction medium, the biosensor response was also monitored at different

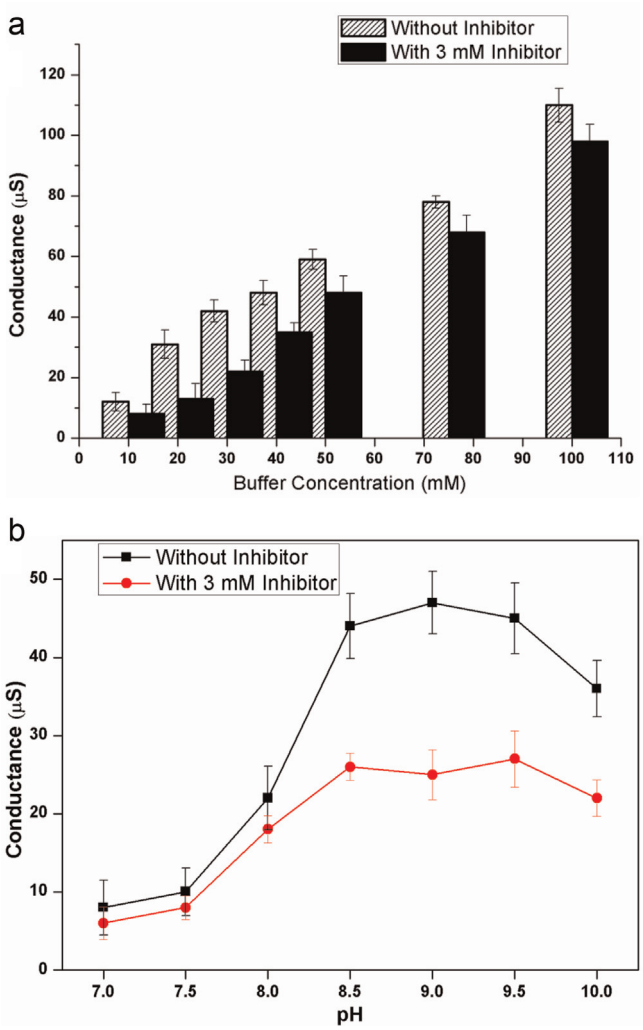

Fig. 3. Effect of varying buffer concentration (a), and pH (b), on the biosensor response in the absence and presence of inhibitor. For both experiments, the biosensor response was measured in Tris HCl buffer (30 mM) at a substrate concentration of 1 mM.

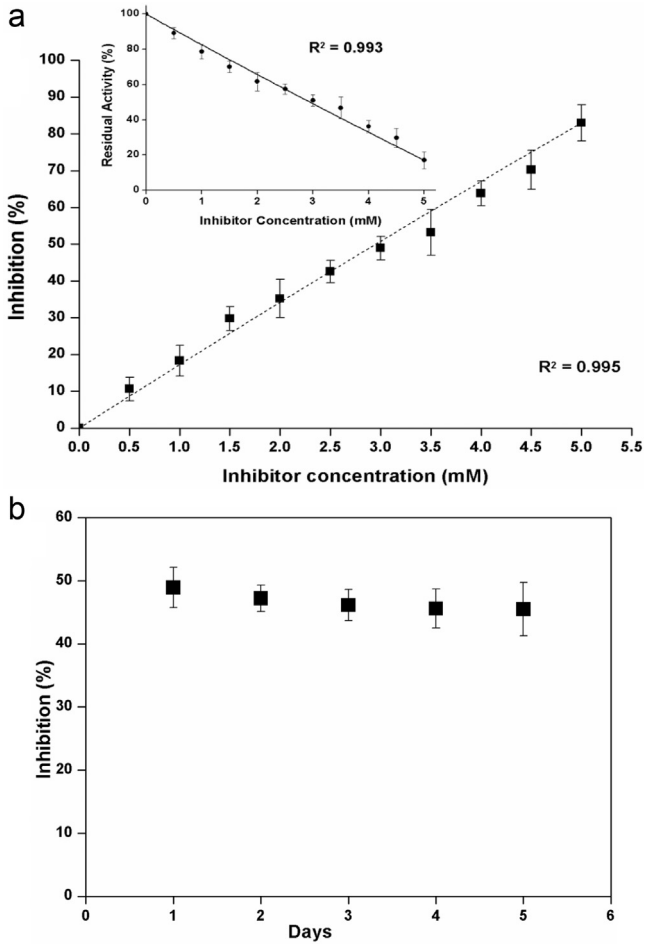

Fig. 4. Calibration curve of alkaline phosphatase biosensor for different concentrations of inhibitor (a). The percentage of APase inhibition was plotted as a function of inhibitor concentration (0.5–5 mM), when measured in Tris HCl buffer (30 mM, pH 9.0) at the substrate concentration of 1 mM. The variations in the biosensor response to 3 mM inhibitor concentration was checked for five successive measurements (b). After each measurement, biosensor was washed with Tris HCl buffer (30 mM, pH 9.0), and stored at 4 °C.

pH. Fig. 3b shows the variation in the biosensor response at varying pH with, and without inhibitor at a substrate concentration of 1 mM. Here, we observed a comparable activity at pH 8.5 to 9.5, with maximum variation in the conductance was obtained at pH 9.0 ($p < 0.05$). So, all further measurements were carried out at a pH of 9.0. For immobilized APase, a range of pH 8.0 to 10.0 has also been reported in the literature (Chouteau et al., 2005; García Sánchez et al., 2003; Mazzei et al., 2004).

3.3. Inhibition curve

Once the optimal conditions were determined, the conductometric measurements were carried out for different concentrations of phosphate. A calibration curve was prepared by plotting the percentage inhibition of enzymatic activity against the varying concentration of phosphate (inhibitor). Fig. 4a shows a linear response of the biosensor in the concentration range of 0.5 mM to 5 mM, with a correlation coefficient (R^2) of 0.995. With every 500 μM addition in the inhibitor concentration, a significant decrement in the enzymatic activity and thus, an increment in the percentage inhibition were obtained. The proposed biosensor have a wider linear range as compared to some multi-enzymatic biosensor reported for phosphate estimation (Mousty et al., 2001; Wollenberger et al., 1992; Zhang et al., 2008). The inhibition rates were considered significant when they exceed 7%. The upper limit of linearity was found to be 5 mM, while the limit of detection (LOD) was determined as 50 μM, equivalent to three times the blank value. Thus, obtained LOD is much lower than the concentration of phosphate found in human blood serum, and also comparatively better than the detection limit of some recently reported amperometric phosphate biosensors (Gilbert et al., 2010; Lawal and Adeloju, 2013).

3.4. Application in real sample analysis

The application of this alkaline phosphatase biosensor in real sample analysis (human blood serum) was also investigated. For this, the serum was isolated from blood and stored at −20 °C, when not in use. Before the analysis, the serum samples were incubated at 56 °C for 30 min to heat inactivate the serum APase (Neale et al., 1965). 5 serum samples from healthy human adults were analyzed by the conventional ammonium-molybdate method (Ramakrishnan and Sulochana, 2012), as well as by the developed biosensor to determine the initial concentration of phosphate, and a percentage of coefficient of variation (%CV) was calculated. A total of 13 samples were then prepared from the previously analyzed healthy human serum samples either by diluting the samples with the buffer or spiked with known concentration

Table 1
Recovery analysis of varying concentrations of serum phosphate by APase conductometric biosensor.

Serum sample	Initial concentration *(mM)	Phosphate concentration Final concentration (mM)	Biosensor measured (mM)	Recovery# (%)
Unspiked				
1	1.32	1.32	1.15 ± 0.25	87
2	1.15	1.15	1.18 ± 0.21	103
3	1.24	1.24	1.09 ± 0.57	88
4	1.28	1.28	1.29 ± 0.74	101
5	1.19	1.19	1.22 ± 0.21	102
Mean	1.24		1.18	
SD	0.06		0.07	
% CV	4.8		5.7	
Spiked				
1	1.32	4.5	4.55 ± 0.22	101
2	1.32	5	5.21 ± 0.77	104
3	1.15	1.4	1.34 ± 0.17	96
4	1.15	1.8	1.62 ± 0.28	90
5	1.24	4.2	4.34 ± 0.16	103
6	1.24	2.4	2.32 ± 0.29	97
7	1.28	2.8	2.59 ± 0.33	92
8	1.28	3.7	3.39 ± 0.37	92
9	1.19	3.5	3.38 ± 0.24	96
10	1.19	2.1	2.17 ± 0.14	103
Diluted				
1	1.32	0.8	0.81 ± 0.21	101
2	1.15	1.0	0.91 ± 0.14	91
3	1.24	0.5	0.43 ± 0.22	86

* As determined by ammonium molybdate method.
# Recovery={concentration of phosphate measured by the biosensor/final concentration of phosphate} × 100 SD—standard deviation; % CV—percentage coefficient of variation.

Table 2
Phosphate recovery from tap water using APase conductometric biosensor.

Tap water sample	Phosphate concentration			Recovery (%)
	Initial concentration (μM)	Spiked concentration (μM)	Biosensor measured (μM)	
1	nd	500	521 ± 8.6	104
2	nd	500	505 ± 12.32	101
3	nd	500	511 ± 8.63	102
Mean			512.33	
SD			6.59	
% CV			1.28	

nd—not determined.
SD—standard deviation; % CV—percentage coefficient of variation.

of phosphates. All thus prepared samples were then tested for phosphate concentration with the help of APase biosensor developed. For blank samples, DI water was added to the reaction mixture containing Tris HCl buffer (30 mM, pH 9.0) and substrate (1.0 mM, pH 9.0). Table 1 shows the different concentrations of serum phosphate analyzed with the APase biosensor. The mean concentration of phosphate found in the serum samples was 1.24 ± 0.06 mM as determined by ammonium molybdate method, while a mean concentration of 1.18 ± 0.07 mM was obtained by the APase biosensor. The phosphate recovery obtained from all the serum samples (spiked, unspiked and diluted) was from 86% to 104%. The small variations in the biosensor response might be due to the presence of other serum components; however the interference is not very significant. A similar concentration of 1.19 ± 0.0085 mM in human serum sample was reported by an amperometric biosensor developed by immobilizing pyruvate oxidase (Rahman et al., 2006). Based on the recovery, we can conclude that the biosensor possesses good anti-interference ability with other blood serum component and thus, can be effectively used for the analysis of phosphate ions in the blood serum.

In addition to serum, the developed biosensor was also used for the analysis of tap water (from lab). As the normal concentration of phosphate present in the tap water is found to be very low, all the samples were spiked with 500 μM phosphate before the analysis. Table 2 shows the phosphate recovery from tap water samples. The mean recovery was 102.3% ($n=3$) for the samples spiked with 500 μM of phosphate, with coefficient of variation of 1.28%. Most of the reported methods for phosphate detection in water samples are based on the *direct determination,* employing pyruvate oxidase. A conductometric biosensor for real water analysis for phosphate detection has been reported with a recovery of 98–113% (Zhang et al., 2008). Pond water spiked with 10 μM of phosphate gave a recovery of 103.2% with percentage coefficient of variation of 19.7%, by a pyruvate oxidase immobilized amperometric biosensor (Gilbert et al., 2011).

3.5. Stability

The reusability of the APase immobilized biosensor was also determined for a known phosphate concentration (3 mM). It could be seen from Fig. 4b that the biosensor response is fairly stable, and almost 93% of the response is maintained after 5 times of reuse. The relative standard deviation of the biosensor response was found to be 7% ($n=5$), which indicate a higher stability of the immobilized alkaline phosphatase in APase conductometric biosensor. The shelf life of the enzyme immobilized biosensor system was found to be 30 days, with a 12% decrement in the biosensor response was observed, when stored at 4 °C in buffer (Tris HCl, pH 9.0) filled condition. However, a multi-enzymatic conductometric biosensor for phosphate detection reported to lost 50% of its initial response after three weeks (Zhang et al., 2008), while a 30% decrement in the sensitivity of phosphate biosensor after two weeks has been reported for a tri-enzymatic biosensor (Mousty et al., 2001).

4. Conclusion

In this study, a simple, novel, and cost-effective conductometric biosensor for the indirect determination of phosphate ions in aqueous solution has been demonstrated. The developed biosensor is based on the inhibition of the activity of alkaline phosphatase, immobilized on the glass internal surface. This enzyme has never been reported to be used solely in the development of biosensor for indirect phosphate estimation. The biosensor showed good performance for phosphate analysis, with a broad detection range (0.5–5 mM), and a lower detection limit (50 μM). With such a higher recovery in the serum samples, the biosensor could certainly be used for the determination of normal and altered level of phosphate in human blood serum, and thus can replace the conventional methods of phosphate estimation in biological fluids.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2015.01.064.

References

Bachan Upadhyay, L.S., Verma, N., 2013. Anal. Lett. 46, 225–241.
Bansal, V.K., 1990. Serum Inorganic Phosphorus, in: Clinical Methods: The History, Physical, and Laboratory Examinations. Butterworths, Boston.
Berezhetskyy, A.L., Sosovska, O.F., Durrieu, C., Chovelon, J.-M., Dzyadevych, S.V., Tran-Minh, C., 2008. IRBM 29, 136–140.
Chouteau, C., Dzyadevych, S., Chovelon, J.-M., Durrieu, C., 2004. Biosens. Bioelectron. 19, 1089–1096.
Chouteau, C., Dzyadevych, S., Durrieu, C., Chovelon, J.-M., 2005. Biosens. Bioelectron. 21, 273–281.
Coburn, S.P., Mahuren, J.D., Jain, M., Zubovic, Y., Wortsman, J., 1998. J. Clin. Endocrinol. Metab. 83, 3951–3957.
Cosnier, S., Gondran, C., Watelet, J.-C., De Giovani, W., Furriel, R.P.M., Leone, F.A., 1998. Anal. Chem. 70, 3952–3956.
Dzyadevych, S.V., Arkhypova, V.N., Korpan, Y.I., El'skaya, A.V., Soldatkin, A.P., Jaffrezic-Renault, N., Martelet, C., 2001. Anal. Chim. Acta 445, 47–55.
Fernley, H.N., Walker, P.G., 1967. Biochem. J. 104, 1011–1018.
García Sánchez, F., Navas Díaz, A., Ramos Peinado, M., Belledone, C., 2003. Anal. Chim. Acta 484, 45–51.
Gilbert, L., Browning, S., Jenkins, A.T.A., Hart, J.P., 2010. Microchim. Acta 170, 331–336.
Gilbert, L., Jenkins, A.T.A., Browning, S., Hart, J.P., 2011. Sens. Actuators B Chem. 160, 1322–1327.
Guilbault, G.G., Nanjo, M., 1975. Anal. Chim. Acta 78, 69–80.
Hanss, M., Rey, A., 1971. Biochim. Biophys. Acta-Enzymol. 227, 618–629.
Lawal, A.T., Adeloju, S.B., 2013. Biosens. Bioelectron. 40, 377–384.
Lawrence, A.J., Moores, G.R., 1972. Eur. J. Biochem. 24, 538–546.
Mazzei, F., Botrè, F., Montilla, S., Pilloton, R., Podestà, E., Botrè, C., 2004. J. Electroanal. Chem. 574, 95–100.
Mousty, C., Cosnier, S., Shan, D., Mu, S., 2001. Anal. Chim. Acta 443, 1–8.
Neale, F.C., Clubb, J.S., Hotchkis, D., Posen, S., 1965. J. Clin. Pathol. 18, 359–363.
Popovtzer, M., Levi, M., 2001. Disorders of Phosphate Balance Atlases of Diseases of the kidney, pp. 7.1–7.14.
Prieto-Simón, B., Campàs, M., Andreescu, S., Marty, J.-L., 2006. Sensors 6, 1161–1186.
Rahman, M.A., Park, D.-S., Chang, S.-C., McNeil, C.J., Shim, Y.-B., 2006. Biosens. Bioelectron. 21, 1116–1124.
Ramakrishnan, S., Sulochana, K., 2012. Manual of Medical Laboratory Techniques. JP Medical Ltd., London, UK.
Su, Y., Mascini, M., 1995. Anal. Lett. 28, 1359–1378.
Sudduth, K.A., Kim, H.-J., Motavalli, P.P., 2014. Soil. In: Moretto, L.M., Kalcher, K. (Eds.), Environmental Analysis by Electrochemical Sensors and Biosensors, Nanostructure Science and Technology. Springer, New York, New York, NY, pp. 23–61.
Torriani, A., 1968. J. Bacteriol. 96, 1200–1207.
Upadhyay, L.S.B., Verma, N., 2014. Process Biochem. 49, 1139–1143.
Walsdorf, S.N.B., Alexandrides, G. 2005. Therapy for kidney disease; complexing phosphorus with calcium glutarate ; prevent forming kidney stones.
Wang, X., Chen, L., Xia, S., Zhu, Z., Zhao, J., Chovelon, J.-M., Jaffrezic-Renault, N., 2006. Int. J. Electrochem. Sci. 1, 55–61.
Wollenberger, U., Schubert, F., Scheller, F.W., 1992. Sens. Actuators B Chem. 7, 412–415.
Zhang, Z., Jaffrezic-Renault, N., Bessueille, F., Leonard, D., Xia, S., Wang, X., Chen, L., Zhao, J., 2008. Anal. Chim. Acta 615, 73–79.
Zhylyak, G.A., Dzyadevich, S.V., Korpan, Y.I., Soldatkin, A.P., El'skaya, A.V., 1995. Sens. Actuators B Chem. 24, 145–148.