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REVIEW

Recent advances in phosphate biosensors

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Abstract A number of biosensors have been developed for phosphate analysis particularly, concerning its negative impact within the environmental and biological systems. Enzymatic biosensors comprising either a single or multiple enzymatic system have been extensively used for the direct and indirect analysis of phosphate ions. Furthermore, some non-enzymatic biosensors, such as affinity-based biosensors, provide an alternative analytical approach with a higher selectivity. This article reviews the recent advances in the field of biosensor developed for phosphate estimation in clinical and environmental samples, concerning the techniques involved, and the sensitivity toward phosphate ions. The biosensors have been classified and discussed on the basis of the number of enzymes used to develop the analytical system, and a comparative analysis has been performed.

Keywords Biosensors · Detection limit · Enzymes · Phosphate · Sensitivity

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Introduction

Phosphorus is the sixth most abundant element in the human body. It is present either as an inorganic phosphate or as an organically-bound phosphate (Berner and Shike 1988). Phosphate is the most abundant intracellular anion which is mainly present in the blood in multiple forms such as phospholipids, HPO_4^{2-}, $H_2PO_4^-$ and free PO_4^{3-}. Phosphate is a key building block for many essential intracellular compounds mainly nucleic acids, phospholipids, enzymes and nucleoproteins (Bugg and Jones 1998). It acts as a precursor for the synthesis of ATP, which is the source of energy for most of the cellular reactions. It also acts as a precursor for 2,3-diphosphoglycerate, which regulates the dissociation of O_2 from hemoglobin (Bunn et al. 1974). It is one of the major factors in the maintenance of bone health, and its deficiency results in the bone pathology and clinical illness (Penido and Alon 2012). In serum, more than 85 % of phosphorus is present as the free ion and less than 15 % is protein-bound (Moe 2006). Any changes in the content or concentration of phosphate modulate the activity of a number of metabolic pathways. Dietary intake and gastrointestinal absorption of phosphorus, urinary excretion of phosphorus, and shifts between the intracellular and extracellular spaces are the major factors that determine the serum phosphorus concentration. Abnormalities in any of these steps can result either in hypophosphatemia or hyperphosphatemia (Levi and Popovtzer 2001).

In the body, the kidney plays a critical role in controlling the phosphate level of blood (Bugg and Jones 1998; Honda et al. 2007). The main role of the kidney in phosphate regulation is that it filters out excess of phosphate in urine through the urinary tract. Since it is the kidney that controls the phosphate level, a high level of body phosphate is generally an indication of kidney-associated problems. Serum phosphate reflects a minor percentage of total body phosphorus, and is present in the form of inorganic phosphate in extracellular fluid (Bansal 1990; Moe 2006). However, this serum phosphate is easily measurable, and indicates the level of body phosphorus. The normal range of phosphate in human serum is 1.1–1.4 mM (Bansal 1990), and any change in its concentration needs to be diagnosed in the early stages as prolonged persistence of altered phosphate levels may have some major consequences. The level of phosphate is also inversely related to the calcium level of blood (Moe 2008). An increment in the phosphate concentration, i.e. hyperphosphatemia, causes hypocalcaemia by precipitating calcium, decreasing vitamin D production, and interfering with parathyroid hormone-mediated bone resorption (Walsdorf and Alexandrides 2005; Musso et al. 2008). On the other side, a decreased phosphate level result into decreased erythrocyte 2,3-diphosphoglycerate levels, which increases the O_2 affinity of hemoglobin, and consequently reduces the O_2 release at the tissue level (MacDonald 1977; Levi and Popovtzer 2001). It also tends to decrease the intracellular ATP level, which may result in the impairment of cell functions which are dependent on energy-rich phosphate compounds (Vorum and Ditzel 2014).

Not only in serum, but measuring the phosphate level in aquatic environments can be a very important tool in understanding the quality of water. Phosphorus is usually present in the natural water as phosphates (orthophosphates, polyphosphates, and organically-bound phosphates) (Nollet and Gelder 2013; Spellman 2013). It is normally required for the growth of aquatic plants but at a very low concentration of 0.025–0.1 mg phosphates/l (Fadiran et al. 2008). However, due to the excessive use of fertilizers, industrial effluents, laundry, human and animal waste, the phosphate level of water bodies increases, which over-fertilizes the aquatic plants, and creates a condition of "eutrophication" (Smith et al. 1999). Eutrophication induces explosive growth of the plants and algae due to oversupply of the nutrients. These plants and algae have a shorter life span, and they die more quickly than they can be decomposed. Their decomposition consumes most of the dissolved O_2, and creates a state of hypoxia (low level of dissolved O_2) which affects the life of other aquatic organisms (Karim et al. 2002; Gubelit and Berezina 2010). The primary limiting factor in the eutrophication process is phosphate (Werner 2009), so measuring phosphate level is a major concern from environmental monitoring point of view.

Thus, a need arises for a fast, safe and economical method for the detection of these phosphate ions in both, biological and environmental samples. In this respect, biosensors can act as useful analytical devices. During the last decades, a number of enzymatic biosensors, both mono and multi-enzymatic have been reported in the literature which has been summarized in this article, concerning the techniques involved and the sensitivity toward phosphate ions.

Conventional methods of phosphate detection

The conventional methods used for the determination of phosphate include a spectrophotometric method (Haemmerli et al. 1990; Ramakrishnan and Sulochana 2012). In this method, inorganic phosphate reacts with ammonium molybdate to produce phospho-molybdate (reduced), which is measured at 340 nm. However, this method is tedious and time consuming (Kwan et al. 2005; Rahman et al. 2006). Moreover, use of some carcinogenic chemicals, such as molybdenum, makes this procedure, health hazardous (Zhang et al. 2008; Gilbert et al. 2011). In addition to this, phosphate sensors in the form of ion selective electrodes have also been developed but these ion selective membranes have a very poor selectivity for the phosphate ions due to its very high hydration energy (Zhang et al. 2008). Also, the free energy of phosphate species is very small, and the large size of phosphate ions interferes with the use of size-exclusion principles for increased selectivity (Tafesse 2014).

Phosphate biosensors

For phosphate analysis, a number of biosensors, specifically enzymatic biosensors, have been developed

in which the phosphate usually acts as the substrate of an enzyme-catalyzed reaction. However, there are few reports of the biosensor based on the indirect estimation of phosphate ions in which the phosphate acts as an inhibitor of the enzyme catalyzed reactions (Guilbault and Nanjo 1975; Cosnier et al. 1998; Upadhyay and Verma 2015) as shown in Fig. 1.

Most commonly used enzymes for the development of phosphate biosensor are pyruvate oxidase, maltose phosphorylase, acid phosphatase, glucose oxidase, mutarotase, alkaline phosphatase, purine nucleoside phosphorylase, xanthine oxidase, horseradish peroxidase, phosphorylase A, phospho-glucomutase and phosphate dehydrogenase. These enzymes are either used singly, as a pair or as multi-enzymes to sequentially catalyze a reaction utilizing the phosphate, either as a substrate or an inhibitor, for its quantitative analysis. Since there are limited reports reviewing the phosphate biosensors, this present article provides an overview of the biosensors reported for the phosphate detection till date. For better understanding, the phosphate biosensors have been classified on the basis of the number of enzymes used to develop the system, and are described in details concerning the reaction involved and other parameters.

Mono-enzymatic biosensors

As compared to bi- or multi-enzymatic biosensor, mono-enzymatic biosensor provides a cost-effective analytical system. Moreover, it involves a simple procedure for the immobilization of enzymes and thus, reduces the interferences from other components of the sample (Ogabiela and Adeloju 2014). For a mono-enzymatic phosphate biosensor system, pyruvate oxidase (POD) is the most widely used enzyme. Pyruvate oxidase (EC 1.2.3.3) is an enzyme that belongs to the family of oxido-reductase, and catalyzed the following reaction:

$$pyruvate + phosphate + O_2 \overset{POD}{\leftrightarrow} acetyl\,phosphate + CO_2 + H_2O_2$$

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

The analytical response of the POD-based biosensor is generally based on the electro-catalytic oxidation of H_2O_2. POD requires the presence of thiamine pyrophosphate (TPP), flavine adenine dinucleotide (FAD) and Mg^{2+} for the catalysis to take place, but these cofactors do not play any role in the selective nature of biosensor (Gavalas and Chaniotakis 2001). The reaction is phosphate-dependent and thus has been applied to develop the biosensor for phosphate estimation in water samples (Kubo et al. 1991; Zhang et al. 2008; Gilbert et al. 2010, 2011; Ogabiela and Adeloju 2014), and biological fluids such as urine (Gilbert et al. 2010, 2011), serum (Gavalas and Chaniotakis 2001; Rahman et al. 2006) and saliva (Kwan et al. 2005). An amperometric biosensor was developed by immobilizing POD on cellulose acetate and cellulose nitrate membranes with the help of glutaraldehyde as a cross-linking agent (Gilbert et al. 2011). The authors reported a lower detection limit (LOD) and sensitivity of 2 μM and 0.406 nA phosphates/μM respectively, with a potential application in determining phosphate in pond water and urine samples. The sensitivity was far better than the sensitivity of an amperometric biosensor developed by immobilizing POD onto the cobalt phthalocyanine-

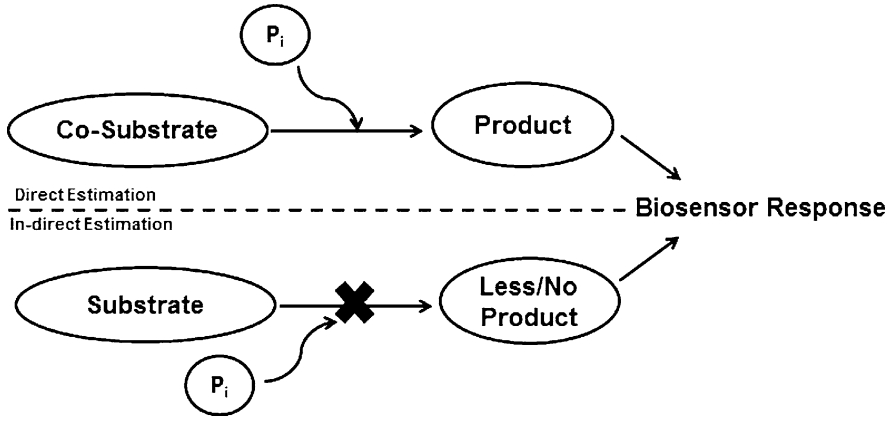

Fig. 1 Schematic representation of the working principle of phosphate (P_i) biosensors

modified, screen-printed carbon electrode in a sandwich format (Gilbert et al. 2010). Also, the response time of the latter biosensor was much higher than the previous biosensor. For human serum, an amperometric biosensor was reported by immobilizing the stabilized POD into a highly porous and conductive carbon electrode through adsorption, with a detection limit and response time of 4.8 μM and 20–40 s, respectively (Gavalas and Chaniotakis 2001). However, to avoid any interference from materials such as ascorbic acid, uric acid, acetaminophenone etc., a pre-oxidizing cell was incorporated in the system for the oxidation of these species. For same samples, a lower detection limit and response time of 0.3 μM and 6 s, respectively, has been reported for the biosensor fabricated by covalent immobilization of POD onto nanoparticle composed conducting polymers (Rahman et al. 2006), with good anti-interference ability to the species mentioned earlier. The advantage of incorporating nanoparticles composed conducting polymers was that it exhibits a high surface area and thus, favors a high enzyme loading. To determine the salivary phosphate, an amperometric biosensor has been reported which was developed by immobilizing pyruvate oxidase on a screen-printed electrode (Kwan et al. 2005). The biosensor showed a linear range from 7.5 to 625 μM, with a detection limit of 3.6 μM. This biosensor, however, achieved rapid determinations of phosphate ions with a slightly higher magnitude when compared with the commercial kits, due to its high viscosity, while lower phosphate content was reported for the filtered samples.

Although, these amperometric biosensors provides a facile approach for phosphate estimation, a proper functioning of these biosensor depends highly on the kinetics of the enzyme transfer process between the prosthetic groups of the enzyme and electrode surface (Habermüller et al. 2000). In the case where an enzyme is directly immobilized on the electrode surface and the detection is based on the monitoring of O_2 concentration, it is difficult to maintain reproducibility of biosensor due to varying O_2 concentrations within the sample (Borgmann et al. 2011). Moreover, the biosensors based on a H_2O_2-based electrode have poor selectivity when used in the biological fluids (Turner and Malhotra 2003). Besides amperometric transduction, a potentiometric biosensor has also been reported which was based on the change in the potential of the reaction medium as a result of pH change after the electroxidation of H_2O_2 (Ogabiela and Adeloju 2014). The biosensor was developed by entrapping the POD enzyme in a polypyrrole film, which can detect phosphate at 3 μM in lake water samples, with good anti-interference ability to Cl^-, SO_4^{2-} and NO_3^-. Recently, we reported a conductometric biosensor for phosphate estimation in biological fluids which is based on the inhibition of alkaline phosphatase by the phosphate ions (Upadhyay and Verma 2015). The biosensor was developed by immobilizing the enzyme on the internal surface of a glass test tube with the help of cysteine functionalized silver nanoparticles (Upadhyay and Verma 2014) and can detect phosphate from 0.5 to 5 mM, with detection limit of 50 μM. Table 1 provides a comparative analysis of some of the mono-enzymatic biosensor reported for phosphate estimation.

Bi-enzymatic biosensors

The two most commonly and widely used enzymes for the development of phosphate biosensors are nucleoside phosphorylase (NP; EC 2.4.2.1) and xanthine oxidase (XOD; EC 1.17.3.2) (Haemmerli et al. 1990; Male and Luong 1991; Kulys et al. 1992; Wollenberger et al. 1992; Lawal and Adeloju 2010, 2013; Adeloju and Lawal 2011). Nucleoside phosphorylase catalyzes the phosphorylation of inosine to ribose 1-phosphate and hypoxanthine, which is then oxidized to uric acid by xanthine oxidase as shown in the following reactions:

$$Inosine + phosphate \overset{NP}{\leftrightarrow} ribose\ 1\text{-}phosphate + hypoxanthine$$

$$hypoxanthine + 2H_2O + O_2 \overset{XOD}{\rightarrow} uricacid + 2H_2O_2$$

The determination of phosphate can thus be achieved by either monitoring the consumption of O_2, or the production of H_2O_2 and/or uric acid during the enzymatic reactions. An amperometric biosensor (Haemmerli et al. 1990) was developed by the co-immobilization of both enzymes (NP-XOD enzyme system) on a polycarbonate membrane mounted on the tips of H_2O_2 and O_2 electrodes. The biosensor had a linear range in 10–250 μM with a response time of 2 min, however the lifetime and stability of the biosensor was unsatisfactory for practical applications.

Table 1 Comparative analysis of mono-enzymatic biosensors for phosphate determination in water samples and biological fluids

Enzyme	Transducer	LOD (μM)	Linear range (μM)	Response time	Application	References
Pyruvate oxidase	Amperometric	0.3	1–100	6 s	Human serum	(Rahman et al. 2006)
		3.6	7.5–625	4 min	Human saliva	(Kwan et al. 2005)
		2	2.5–130	13 s ± 1	Pond water and human urine	(Gilbert et al. 2011)
		4.8	50–1250	20–40 s	Human serum	(Gavalas and Chaniotakis 2001)
		100	363–1000	4.9 min	Urine and water	(Gilbert et al. 2010)
		12	12–80	7 min	Water	(Kubo et al. 1991)
	Potentiometric	3	15–400	–	Lake water	(Ogabiela and Adeloju 2014)
Maltose phosphorylase	Conductometric	1.0	1.0–20, and 20–400	10 s	Water	(Zhang et al. 2008)
Alkaline phosphatase	Conductometric	50	0.5–5.0	20 min	Human serum and tap water	(Upadhyay and Verma 2015)

To further improve the biosensor characteristics such as stability, detection limit and reproducibility, flow injection analysis (FIA) system has been introduced. A FIA amperometric biosensor for phosphate estimation in the food products and plasma has been reported (Male and Luong 1991). The response of the biosensor was linear up to 100 μM with a lower detection limit of 1.25 μM, and the system could be used for 160 repeated analyses with periodic calibration. A highly sensitive biosensor which can detect phosphate in nano-molar range was developed by immobilizing the enzymes on a gelatin membrane and fixed on a Clark-type O_2 electrode (Wollenberger et al. 1992). The biosensor had a detection limit of 25 nM with a linear response in the range 0.5–100 μM, however; the biosensor was very unstable and lost its response within 4 days.

Not only amperometric but some potentiometric biosensors have also been reported recently for the phosphate analysis (Lawal and Adeloju 2010, 2013; Adeloju and Lawal 2011). A comparative study was done for the amperometric and potentiometric biosensor for phosphate analysis by immobilizing NP-XOD via entrapment into polypyrrole films (Lawal and Adeloju 2013). A minimum of 20 μM phosphate could be sensed by the potentiometric biosensor, while amperometric biosensor could detect a minimum of 0.1 mM phosphate. The same research group has previously reported a detection limit of 1 μM for the potentiometric biosensor used for detecting the phosphate level in polluted water (Lawal and Adeloju 2010). However, there is one problem in using inosine as a substrate in bi-enzymatic biosensor for phosphate estimation. Inosine is very unstable, and therefore the system may not be suitable for monitoring the phosphate level of natural waters (Nakamura et al. 1997).

Some other enzyme systems comprising alkaline phosphatase-glucose oxidase and alkaline phosphatase-polyphenol oxidase has also been reported for phosphate estimation. Alkaline phosphatase (ALP, EC 3.1.3.1) and glucose oxidase (GOD, EC 1.1.3.4) sensor system for phosphate analysis utilized the following reactions:

$$\beta\text{-D-}glucose\,6\text{-}phosphate + H_2O \overset{ALP}{\rightarrow} \beta\text{-D-}glucose + orthophosphate$$

$$\beta\text{-D-}glucose \overset{GOD}{\rightarrow} H_2O_2 + gluconic\,acid$$

While the reaction catalyzed by the alkaline phosphatase and polyphenol oxidase (POD) enzyme system is as follows:

$$phenyl\,phosphate + H_2O \overset{ALP}{\rightarrow} phenol + orthophosphate$$

$$phenol + O_2 \overset{POD}{\rightarrow} o - quinone + H_2O$$

In both biosensing systems, the phosphate acts as an inhibitor of ALP activity and thus, the phosphate

concentration is inversely proportional to the rate of enzyme catalyzed reaction. The ALP-GOD enzyme system was the first system reported to be used for the assay of phosphate ions in the range of 0.1–1 mM (Guilbault and Nanjo 1975). This enzyme electrode also suitable for the analysis of oxyacids such as arsenate, tungstate, molybdate and borate; however its sensitivity was very low (0.1 mM). In another approach, the ALP-GOD system was immobilized on an Immobilon membrane, and was used to determine the phosphate in some synthetic and real samples with a detection limit of 4 μM (Su and Mascini 1995). A bi-enzyme electrode utilizing ALP-POD enzyme system immobilized in polypyrrole film has been reported for the amperometric determination of phosphate (Cosnier et al. 1998). The sensitivity and the detection limits were reported to be 1.27 mA/M.cm^2 and 2 μM, respectively.

To increase the sensitivity, chemiluminescence-based biosensors have been reported and widely used for the phosphate analysis (Ikebukuro et al. 1996a, b; Nakamura et al. 1997, 1999b). Chemiluminescence is a phenomenon in which light is produced as a result of some chemical reactions. The H_2O_2 generated by the pyruvate oxidase reaction is allowed to react with the luminol, a reaction catalyzed by the horse radish peroxidase (HRP, EC 1.11.1.7), to produce chemiluminescence. Chemiluminescence based biosensors generally utilized the following chemical reactions:

$$pyruvate + phosphate + O_2 \overset{POD}{\leftrightarrow} acetyl\,phosphate + CO_2 + H_2O_2$$

$$luminol + H_2O_2 + 2OH^- \overset{HRP}{\rightarrow} N_2 + 4H_2O + h\upsilon$$

Initially, a flow injection analysis system for the rapid determination of phosphate was developed by immobilizing pyruvate oxidase on amino-alkylated controlled pore glass (CPG) packed to a column, and connected to a luminometer (Ikebukuro et al. 1996b). The developed sensor showed a linear response in the range of 4.8–160 μM phosphate, with a response time and detection limit of 3 min and 2.9 μM, respectively. However, the reported biosensor was not useful to detect the phosphate level of river water as the maximum permissible concentration was 0.32 μM. To further improve the sensitivity of FIA system, they placed the detection unit directly in front of the immobilized peroxidase. With such system, they reported a detection limit of 74 nM which was sufficient to determine the maximum permissible concentration of natural waters of Japan (Ikebukuro et al. 1996a).

Due to the high sensitivity of *Arthromyces ramosus* peroxidase (ARP) than HRP in FIA system, an automatic system comprising chitopearl beads immobilized pyruvate oxidase and ARP-luminol, for the determination of phosphate ion has been reported (Nakamura et al. 1997). It had a detection limit and linear range of 0.16 μM and 0.16–32 μM, respectively, with positive application in dam water analysis. However, the authors did not perform any interference studies caused by any reducing species dissolved in the dam water. In another approach, the same researchers used puruvate oxidase G from *Aerococcus viridans*, and immobilized it on *N*-hydroxysuccinicacidimido beads without any cross-linking agent (Nakamura et al. 1999b). This trial sensor system was able to detect phosphate in nanomolar concentration with a linear range from 96 nM to 32 μM. The biosensor response in the presence of some dissolved substances such as metal ions, heavy metals, inorganic and organic compounds was also investigated, and concluded that the treatment of water samples with activated carbon could decrease the interferences to some extent.

Although, most of the reported biosensors for phosphate estimation are bi-enzymatic in configuration, but there are some limitations. The most common disadvantages is that the optimal operating conditions for both the enzymes might be different which further lowers the stability of phosphate biosensor (Rahman et al. 2006). Table 2 provides an overview of some of the bi-enzymatic biosensor developed for phosphate estimation.

Multi-enzymatic biosensor

In amperometric biosensor, the function of enzymatic reaction is to produce or consume electrochemically active species, but only a few enzymes have the ability to produce these redox-active compounds. This further limits the number of analytes that can be measured by mono-enzymatic electrodes, however this problem can be overcome by coupling of different enzymes in sequential or cyclic pathways to configure a multi-

Table 2 Analytical characteristics of Bi-enzymatic biosensors reported for phosphate estimation

Enzyme	Transducer	LOD	Linear range	Response time	Application	References
Nucleoside phosphorylase and xanthine oxidase	Amperometric	0.22 mM	–	1 min	–	(Kulys et al. 1992)
		1.25 μM	Up to 100 μM	5–6 min	Food products and plasma	(Male and Luong 1991)
		25 nM	0.5–100 μM	90 s	–	(Wollenberger et al. 1992)
		–	10–250 μM	2 min	–	(Haemmerli et al. 1990)
	Potentiometric	20 μM	40–120 μM	–	–	(Adeloju and Lawal 2011)
		1.0 μM	5–25 μM	–	Water	(Lawal and Adeloju 2010)
	Amperometric (A) and Potentiometric (P)	0.1 mM (A) 20 μM (P)	0.1–1 mM (A) 20–200 μM (P)	–	Water	(Lawal and Adeloju 2013)
Alkaline Phosphatase and Glucose oxidase	Amperometric	4 μM	8–110 μM and 0.1–1.0 mM	–	Synthetic and real samples	(Su and Mascini 1995)
Alkaline Phosphatase and Polyphenol Oxidase	Amperometric	2 μM	–	–	–	(Cosnier et al. 1998)
Pyruvate Oxidase and HRP	Chemiluminescence	0.16 μM	0.16–32 μM	13.5 min/cycle	Dam water	(Nakamura et al. 1997)
		96 nM	96 nM–32 μM	2 min	River water	(Nakamura et al. 1999b)
		2.9 μM	4.8–160 μM	3 min	River water	(Ikebukuro et al. 1996b)
		74 nM	0.37–7.4 μM	3 min	River water	(Ikebukuro et al. 1996a)

enzymatic biosensor (Wollenberger et al. 1993). For phosphate estimation, few multi-enzymatic biosensors have also been reported employing three or four enzymes (Conrath et al. 1995; Fernández et al. 1998; Nakamura et al. 1999a; Mousty et al. 2001). Tri-enzymatic biosensors relies on the enzymes such as maltose phosphorylase (MP, EC 2.4.1.8), mutarotase (MR, EC 5.1.3.3.) and glucose oxidase (GOD, EC 1.1.3.4) to carry out the reaction in a sequential way which is as follows:

$$maltose + phosphate \overset{MP}{\leftrightarrow} \alpha\text{-D-}glucose + \beta\text{-D-}glucose\ 1\text{-}phosphate$$

$$\alpha\text{-D-}glucose \overset{MR}{\leftrightarrow} \beta\text{-D-}glucose$$

$$\beta\text{-D-}glucose + O_2 + H_2O \overset{GOD}{\leftrightarrow} H_2O_2 + Gluconic\ acid$$

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

The first two reactions generate two molecules of glucose which are then oxidized by glucose oxidase to generate H_2O_2. An amperometric biosensor for phosphate estimation has been fabricated by entrapping these three enzymes (MP, MR and GOD) in laponite clay matrix (Mousty et al. 2001). The laponite clay provides a rapid and easy immobilization procedure with a high hydrophilic character. The authors reported a linear range of 1-50 μM with a sensitivity of 52.4 mA/M/cm^2. H_2O_2 generation could also be

monitored by a chemiluminescence detector after its mixing with luminol. A chemiluminescence flow-injection analysis biosensor has been reported by employing a MP-MR-GOD reaction system combined with an *Arthromyces ramosus* peroxidase-luminol reaction system (Nakamura et al. 1999a). It had a linear range of 10–30 μM with a detection limit of 1 μM, with application in environmental monitoring of river water and marshes. In a different approach, a tri-enzymatic reagent less biosensor has been developed by the enzymes phosphorylase A (PA, EC 2.4.1.1), phosphoglucomutase (PGM, EC 5.4.2.2) and glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49). The enzyme phosphorylase A generates glucose 1-phosphate from glycogen and phosphate, which is then transformed to glucose 6-phosphate by the enzyme phosphoglucomutase. Glucose 6-phosphate is finally oxidized by glucose-6-phosphate dehydrogenase to produce NADH. This NADH is subsequently oxidized by a mediator, osmium(1,10-phenanthroline-5,6-dione)$_2$Cl$_2$ on the electrode surface to generate the current, proportional to the concentration of phosphate (Fernández et al. 1998). The developed biosensor showed a linear response up to 250 mM, with a detection limit and response time of 2 mM and 6 min, respectively.

This strategy was novel in terms of different phosphate co-substrate used, *i.e.* glycogen, and was based on the reversible electrocatalytic recycling of the cofactor. A highly sensitive four enzyme amperometric biosensor system comprising maltose phosphorylase, acid phosphatase, mutarotase and glucose oxidase has also been reported consisting of a recycling and amplifying system (Conrath et al. 1995). The acid phosphatase hydrolyses the phosphomonoester to another mol of glucose and phosphate, and thus can be recycled several times. However, this biosensor lacked sufficient reproducibility, analyzed a lower range of phosphates (0.1–1 μM), and unsuitable for real applications (Nakamura et al. 1999a).

Similar to the bi-enzymatic biosensor, these multi-enzymatic biosensors can also be associated with the disadvantages of having lower stability due to varying optimal conditions of different enzymes and thus, caused fluctuations in the biosensor performances (Kubo et al. 1991; Zhang et al. 2008). Moreover, multi-enzymatic system often leads to more non-specific response due to the presence of substrates of the next enzymes, and are generally expensive (Zhang et al. 2008). Table 3 summarizes different multi-enzymatic biosensor reported for phosphate estimation.

Non-enzymatic biosensors

In addition to the enzymatic biosensors, few biosensors have been reported in which phosphate-binding proteins were used as the bio-recognition element rather than enzyme system, for phosphate estimation (Salins et al. 2004; Okoh et al. 2006; Basheer et al. 2011). In unicellular eukaryotes, such as yeast, and Gram-negative bacteria, such as *E. coli* and *Salmonella* spp., the transport of ligands across the membrane is mediated by some specific proteins, called as periplasmic-binding proteins (PBPs) that bind to the ligand with high affinity (Herrou and Crosson 2013). For phosphate uptake, there is a specific phosphate-specific transport (PST) system (Rosenberg et al. 1977), which is specific for the inorganic form of phosphate (Willsky and Malamy 1980; Salins et al. 2004), and this PST system has been further exploited to construct a biosensor for phosphate analysis. A fluorescence-based biosensor based on the phosphate-binding protein of *E. coli* has been reported in which the single polypeptide chain of PBP was entrapped behind a dialysis membrane at the tip of a bifurcated fiber optic bundle (Salins et al. 2004). The PBP was folded into two similar domains, connected by three short peptide segments that serve as a flexible hinge. In the absence of phosphate, the two domains remain far apart while the presence of phosphate induces a hinge motion in PBP, and the resulting conformational changes are then detected. The reported biosensor could detect the phosphate at a minimum concentration of 1.8 μM; however, the system requires a longer incubation time of 15 min. A label-free biosensor for direct determination of phosphate has been reported in which the high affinity Pho84 plasma membrane phosphate/proton symporter of *Saccharomyces cerevisiae* was immobilized on a self-assembled monolayer on a capacitance electrode, without involving lipid membrane (Basheer et al. 2011). This label free biosensor which was based on the change in capacitance after the binding of phosphate showed a linear response in the range of 5-25 μM, but still the response time was higher.

To increase the selectivity and stability of an analytical system, molecular imprinting technique is

Table 3 Multi-enzymatic biosensors for phosphate determination with comparative analysis of different parameters

Enzyme	Transducer	LOD	Linear range	Response time	Application	References
Maltose phosphorylase, Mutarotase and Glucose oxidase	Amperometric	1 μM	1–50 μM	–	Surface water	(Mousty et al. 2001)
Phosphorylase A, Phosphoglucomutase and Glucose 6-phosphate dehydrogenase	Amperometric	2 mM	250 mM	6 min	–	(Fernández et al. 1998)
Maltose phosphorylase, Acid phosphatase, Glucose oxidase and Mutarotase	Amperometric	10^{-8} M	0.1–1 μM	3 min	Water	(Conrath et al. 1995)
Maltose phosphorylase, Mutarotase, Glucose-oxidase and Peroxidase	Chemiluminescence	1 μM	10–30 μM	3 min	River water	(Nakamura et al. 1999a)

widely used to produce synthetic receptors for different molecules, including phosphates (Kugimiya and Takei 2006, 2008). In this technique, a mixture of target molecules (template) and a monomer are polymerized, with subsequent removal of the template which results in the formation of a cavity to allow binding of a target analyte (Kugimiya and Takei 2006). For phosphate ions, a number of molecularly imprinted polymers (MIPs) based on thiourea groups have been developed, but their application in designing phosphate biosensors is still lagging (Warwick et al. 2013). However, some other analytes having a molecular weight similar to the phosphate have been recently reported to be successfully determined by these MIPs based sensors (Ratautaite et al. 2014).

Conclusion

Despite the ever-increasing number of biosensor with wider applications, reports of phosphate biosensors are still limited. There is no any single biosensor which is commercially available in the market for the phosphate detection. They are still lagging in terms of sensitivity, detection limit, while some of them have a low lifetime and stability in practical applications. Most of the reported biosensors are capable of detecting phosphate only in a confined geographical area. Further, most of the reported phosphate biosensors utilized only the enzymatic recognition mode, where the phosphate acts as the substrate or inhibitor. These enzymatic biosensors, mainly enzyme inhibition-based biosensors for phosphate estimation, generally suffer with low selectivity, particularly when the sample is a multi-analyte. However, this problem can be resolved by employing a protective charged layer, but it further limits the biosensor response. The presence of some electro-oxidizable species such as ascorbic acid, uric acid, Cl^-, SO_4^{2-} etc. in the real samples interferes with the selectivity of the amperometric biosensors. This further hinders the commercialization of the biosensor at large scale.

Affinity-based biosensors provide a different and interesting approach but this methodology has so far not been applied extensively in the construction of biosensor due to some limitations such as loss of functional structure during isolation and purification of membrane proteins. Moreover, these affinity-based biosensors also have a higher analysis/response time as compared to amperometric biosensors. Therefore, a need still arises to design and develop the biosensor exploiting other recognition methods and transducers, suitable for both lab analysis and on-site analysis, and thus, will be helpful in replacing the conventional methods of phosphate analysis. A promising approach to increase the selectivity and stability of phosphate biosensor is molecular imprinting technique. Different immobilization techniques can be implemented with phosphate specific MIPs on different transducer surface for the development of a highly selective phosphate biosensor Moreover, incorporation of nanotechnology in phosphate biosensor designing may also be helpful in improving its sensitivity and performance. Use of self-assembled monolayers as a biosensor interface provides a molecular level control which further opens the opportunities for the development of new transduction mechanism in the biosensor and thus, will be helpful in the fabrication of more precise biosensors.

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