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Review

Determination of inorganic phosphate by electroanalytical methods: A review

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ABSTRACT

Determination of inorganic phosphate is of very high importance in environmental and health care applications. Hence knowledge of suitable analytical techniques available for phosphate sensing for different applications becomes essential. Electrochemical methods for determining inorganic phosphate have several advantages over other common techniques, including detection selectivity, stability and relative environmental insensitivity of electroactive labels. The different electrochemical sensing strategies adopted for the determination of phosphate using selective ionophores are discussed in this review. The various sensing strategies are classified based on the electrochemical detection techniques used viz., potentiometry, voltammetry, amperometry, unconventional electrochemical methods etc., The enzymatic sensing of phosphate coupled with electrochemical detection is also included. Various electroanalytical methods available in the literature are assessed for their merits in terms of selectivity, simplicity, miniaturisation, adaptability and suitability for field measurements.

Contents

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1. Introduction

The determination of phosphate species in environmental samples provides essential data for monitoring the health of ecosystems, investigating biogeochemical processes and for checking compliance with legislation [1]. The presence of inorganic phosphate derived from fertilisers, similarly to nitrates, leads to an excessive growth (eutrophication) of aquatic plants and algae that disrupts aquatic life cycles, while sodium and potassium organophosphate compounds are among the most used pesticides in many intensive agricultural activities and are often found in ground waters, leading to severe health problems [2]. From the perspective of biology, phosphate is one of the most important electrolytes and an essential component of all living organisms. Phosphate plays an important role in biological processes like synthesis of ATP, DNA, and control of pH in blood or lymph fluid. In a clinical setting, phosphate level in serum is determined as part of a routine blood analysis. A knowledge of phosphate level in body fluids can provide useful information about several diseases such as hyperparathyroidism, vitamin D deficiency, and Fanconi syndrome [3]. Analysis of salivary phosphate is considered as a biomarker for different diagnostic tests. The concentration fluctuations of salivary phosphate have been investigated as indicators of ovulation of women, uremic state, and risk of development of dental caries and formation of dental calculus [4].

Another field where phosphate control is assuming an increasing importance is the protection of the cultural heritage. It was hypothesised that phosphate plays a major role in biodeterioration of archaeological sites caused by cyanobacterial biofilms [5]. The concentration of phosphorus varies from 0.2 to 10 mg L^{-1} (6.4×10^{-6} mol L^{-1} to 0.3×10^{-4} mol L^{-1}) in natural and waste waters and from 0.2 to 50 mg kg^{-1} (6.4×10^{-6} mol kg^{-1} to 1.5×10^{-4} mol kg^{-1}) in soil. A maximum permissible concentration of phosphate in river water is 0.32×10^{-6} mol L^{-1} (9.8 μg L^{-1}) and ranges from 0.0143 to 0.143×10^{-3} mol L^{-1} (0.4418 mg L^{-1} to 4.418 mg L^{-1}) in waste water. As a diagnostic fluid, the concentration of phosphate ions in human saliva is found to vary from 5 to 14×10^{-3} mol L^{-1} (154.5 mg L^{-1} to 432.6 mg L^{-1}) [6,7]. It is in the range of $0.81–1.45 \times 10^{-3}$ mol L^{-1} (25.029–43.26 mg L^{-1}) PO_4^{3-} in adult human serum [8,9]. Several analytical methods are being evaluated to measure phosphate in clinical, environmental, industrial, and biological samples. The objective is to develop methods for better detection limits, better sensitivity, and negligible interference from real sample matrix, optimum analysis cost and fast response for phosphate analysis. Different analytical methods such as chromatography, optical fluorescent and colorimetric based (sensing) and electrochemical methods are normally being developed [10–22]. Electrochemical methods have several advantages over the other common methods. The advantages include detection selectivity, stability and relative environmental insensitivity of electroactive labels. Further, spectrophotometric methods involve the addition of many reagents and extraction into organic solvent is often required. Furthermore electrochemical techniques allow miniaturisation and operational simplicity which are highly desirable attributes for field measurements.

Field-based measurements provide a versatile and indeed potentially invaluable screening option for monitoring inorganic phosphate ions for ecological surveys. Interest in the use of field-based measurements stems from a need to provide quick on-site assessments that could cover a greater geographical spread while obviating much of the costs, time delays and loss of sample integrity associated with traditional laboratory-based analysis. While a variety of colorimetric spot test kits are commercially available and possess supreme portability, they can be prone to interference and provide, at best, qualitative results [14]. The need for quick and quantitative field measurements that can be carried out by non-expert investigators could be addressed by the use of electrochemical detection methods [10,23–25]. The extrapolation of such technologies to yield a viable platform for field testing of phosphate appear feasible but issues of selectivity and sensitivity must be clarified. With the advent of wireless sensor networks, the idea of remote sensing is becoming popular and electrochemistry can offer solutions to remote sensing of phosphate ions in environmental samples [26]. Further, electrochemical methods are advantageous for biological diagnostic tests. When one looks into the development of glucose sensors for diabetes management, it is understood that today, the majority of the 6 billion annual assays performed by self-monitoring diabetic people are electrochemical. Further, continuous amperometric monitoring of glucose is nowadays attempted using implanted long term glucose monitors, systems with subcutaneous ultra filtration and micro dialysis fibers coupled to externally worn sensors and reverse-iontophoretic systems [27]. These developments convey the importance of electroanalytical techniques for in vivo sensing. Considering the importance of phosphate in environmental and clinical sectors, it appears worthwhile to write a review comprising the electrochemical approaches available for sensing phosphate. The reviews available to date mostly deal with sample collections, preservation and quality assurance issues of phosphate sensing [1,28]. One comprehensive review covers the bioelectroanalytical aspects of phosphate sensing [14].

Compilation of the existing electroanalytical techniques, will help the researchers to analyse the pros and cons of the currently practised methods. This article will review the existing electroanalytical techniques for their merits in terms of selectivity, simplicity, miniaturisation, adaptability and suitability for field measurements and will address the possibility of interferences from other anions.

Some of the strategies [1,3,6,29–36] that have been applied to the electrochemical detection of phosphate anions are summarised as follows:

- Extraction of the phosphate anion into an inert membrane (e.g. polyvinyl chloride (PVC) membrane) by a non-redoxactive host (e.g. cationic polymers) followed by the detection of the resulting membrane potential. This forms the basis of ion-selective electrodes (ISEs), and chemically modified field-effect transistors (CHEMFETs).
- Detection of the current/potential perturbation response of a redox-active host on complex formation (voltammetric/amperometric). Examples of such hosts include metallocenes/porphyrins/pyrroles bound to a receptor group for phosphate or metal complexes, in which the coordinated metal centre shows an unsaturated coordination environment and thus can bind phosphate via classical coordination chemistry.
- Investigation of electroluminescence properties of dyes like rhodamine when they bind to molybdophosphates.
- Optoelectrochemical detection based on the transmittance changes induced by the adsorption of phosphate on ITO (indium–tin oxide) electrodes under constant applied potential.
- Indirect sensing of phosphate by observing reduction in the catalytic current for the oxidation of glucose on a catalytic electrode (e.g. $(NiOH)_2$/NiOOH electrodes).
- Investigation of blocking of ferrocyanide electron transfer kinetics induced by phosphate anions on gold electrodes modified by self-assembled monolayers of thiols.
- Phosphate sensing based on facilitated ion transfer across liquid/liquid interfaces.
- Electroanalytical sensing of phosphate in the presence of enzymes sensitive to phosphate.
- Mass changes associated with different concentration of phosphate during the electropolymerisation of ethylenedioxythiophene monomer.

2. Potentiometric detection of phosphate

2.1. Potentiometric metal/metal phosphate sensors

One of the early examples of a direct phosphate sensor was based on silver phosphate as the electroactive material. Unfortunately, this ISE method suffers from severe chloride interference [37]. There are several reports on the indirect potentiometric determination of phosphate using various ISEs where the activity of the electroactive cation changes because of the chemical reaction of phosphate with the cation of the active material of the ISE. A cadmium ISE has been used for the determination of phosphate using flow injection potentiometry (FIP) [38]. This method relies on detecting the decrease in $[Cd^{2+}]$ accompanying the formation of $Cd_3(PO_4)_2$ in the carrier stream. This technique is susceptible to interferences from anions that also form insoluble compounds with cadmium. Assaying of phosphate using continuous flow analysis in conjunction with a lead ISE [39], has also been carried out. This approach is based on detecting the ensuing decrease in lead concentration following the precipitation of $Pb_3(PO_4)_2$. Unfortunately, this method also experiences severe interferences due to chloride and calcium, which are among the major constituents in natural waters. A ISE that has demonstrated some success for phosphate determination is based on cobalt/cobalt oxide [40–44]. The response mechanism is subject to some debate, being either a host–guest relationship [40] or a mixed potential response resulting from the slow oxidation of cobalt and simultaneous reduction of both oxygen and Co^{2+} at the surface of the electrode. In addition to response to phosphate, the cobalt electrode is found to respond also to changes in the partial pressure of oxygen in the sample solution. Nevertheless, it has been shown to be capable of detecting phosphate to 0.1 ppm whilst retaining a high degree of selectivity. The following reactions occurring at different pH form the basis of sensing phosphate potentiometrically.

$$3CoO + 2H_2PO_4^- + 2H^+ \rightleftharpoons Co_3(PO_4)_2 + 3H_2O \text{ at pH } 4.0 \quad (1)$$

$$3CoO + 2HPO_4^{2-} + H_2O \rightleftharpoons Co_3(PO_4)_2 + 4OH^- \text{ at pH } 8.0 \quad (2)$$

$$3CoO + 2PO_4^{3-} + 3H_2O \rightleftharpoons Co_3(PO_4)_2 + 6OH^- \text{ at pH } 11.0 \quad (3)$$

2.2. Potentiometric studies based on organotin complexes

Another class of compounds used for phosphate detection is organotin complexes which respond directly to dibasic phosphate [45]. The idea of using organic tin(IV) compounds for phosphate selective electrodes was borrowed from the observation that triphenyltin compounds are good reagents for phosphate extraction [46]. Initial studies with triphenyltin dihydrogenphosphate as carrier resulted in a quite good sensitivity but poor selectivities and very slow responses [47]. Mostly, phosphate selective organotin compounds have only two organic substituents on the tin centre. In contrast, trialkyltin carriers are often selective for Cl^-. Dialkyltin dinitrate ionophores were reported to give the required selectivity pattern, with a slight preference for HPO_4^{2-} over $H_2PO_4^-$. With the increase in the length of the alkyl chain, the interference due to other anions decreases [48,49]. A drawback with respect to these electrodes is their positive responses towards arsenate [49,50]. The tin(IV) centres facilitate binding of the oxygen atoms of the phosphate to the organic complex by withdrawing electrons from the tin. This electron withdrawing property, and consequent phosphate selectivity, could be further increased by replacing alkyltin compounds with benzyltin. Hence dibenzyltin dichlorides were suggested as carriers for HPO_4^{2-} [51,52]. The investigation of dibenzyltin dichlorides with several substituents in the para position of the benzene ring indicated an increase of the phosphate selectivity [53,54]. Highly hydrophilic tribasic citrate is found to interfere in the case of electrodes based on bis(*p*-chlorobenzyl)tin or bis(*p*-fluorobenzyl)tin carriers. However the response mechanism of these electrodes to tribasic citrate and phosphate appears to be different. The severe limitation of all dibenzyltin electrodes is their functional lifetime, which is limited by degradation of the response within days. A multidentate carrier with four tin centres exhibited excellent selectivity to phosphate. However the lifetime of the electrode was less than one day [55]. Some of the ISEs based on distannyl derivatives exhibited good selectivity to phosphate. However, information about their lifetime has not been reported [56]. The chemical structures of the tin based ionophores are given in Fig. 1.

2.3. Potentiometric metal complex sensors

One of the interesting systems based on metal complexes that can recognise phosphate anions is based on Zn .Two Zn(II) – dinuclear systems were studied as receptors for phosphates which were obtained by using two polyamino-phenolic ligands. The affinity of the metalloreceptors towards phosphate sensing was evaluated in aqueous solution in a wide range of pH ($6 < pH < 10$). One of the metalloreceptors was able to selectively discriminate phosphate from pyrophosphate, and on the contrary another receptor exhibited

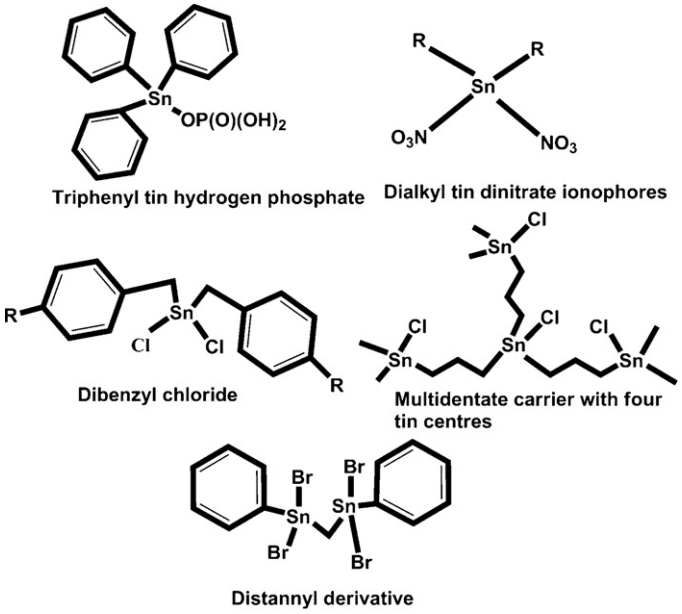

Fig. 1. Tin based ionophores reported in the literature for sensing phosphate.

opposite selectivity. The difference in the selectivity is ascribed to the different Zn(II)—Zn(II) distances between the two metal centres. The potentiometric results have been substantiated by studying the interactions of phosphate with the Zn complexes through NMR and fluorescence measurements [30]. Cobalt phthalocyanine complex was used as an ionophore for phosphate, which gave interesting selectivities [57,58]. ISEs with membranes containing mixed ligand Ni(II) complexes (Ni[dike][diam] where dike = β-diketonate, diam = *N,N′*-di-, tri-, or tetra-alkylated ethylenediamine) were selective to phosphate with response slopes of −21 mV decade^{-1} [59,60].

2.4. Phosphate ISEs based on salophens

Uranyl and vanadyl salophens are used as phosphate ionophores in ion selective membranes which exhibit tolerable phosphate selectivity. Their inadequate stability and short lifetime has forbidden their application in direct use for environmental and clinical analysis. Moreover, they are only functional under strict laboratory conditions. To circumvent these problems efforts have been made to prepare terthiophene monomer appended uranyl-salophen, followed by polymerising its modified monomers to produce functionalised conducting polymer films (CP-ISE). The CP-ISEs showed better electrochemical properties (response time, Nernstian slope and selectivity) for monohydrogenphosphate over conventional ISEs incorporated with the same ionophore. Furthermore, we can resort to miniaturisation with the CP-ISEs since they did not require plasticised-PVC membranes with internal solutions which are needed in the conventional ISEs. The CP-based membrane exhibited excellent functional properties for the ion-to-electron transducers and provided ion-recognition sites for the selective complexation in solid-state ISEs. However this method also was not very successful due to the short life of the sensor [61].

2.5. Potentiometric sensors based on polyamines, guanidinium and ammonium receptors for phosphate

Polyamines form a special group of phosphate carriers because they have no metal centre [62]. Among four macrocyclic polyamines, a macrocycle with one secondary amine and two lactam groups was claimed to give the highest selectivity for phosphate, giving a Nernstian response down to 10^{-6} M HPO_4^{2-}. Another group of workers have used the same polyamine and have demonstrated phosphate sensing in macro and microelectrodes [63].

A zwitterionic bis(guanidinium) ionophore bearing an anionic closo-borane cluster which can complex and selectively extract oxoanions has been investigated in polymeric membrane ISEs. By systematic variation of the concentration of the ion-exchanger sites in the membrane, a reasonably good selectivity for monohydrogenphosphate was obtained. A detection limit of 8.7×10^{-8} M has been reported [64].

The design and synthesis of receptors containing a Cu(II) binding site with appended ammonium groups and guanidinium groups, along with thermodynamic analyses of anion binding, are reported. Both receptors show high affinities (10^4 M^{-1}) and selectivities for phosphate over other anions in 98:2 water:methanol at biological pH. However the authors have not used these compounds in ISEs [65,66]. Table 1 provides the performance of some potentiometric sensors in terms of analytical parameters like detection limit, sensitivity, response time storage life, Nernstian slope value etc.

2.6. Innovative modifications of potentiometric analysis

Established history of potentiometric sensors, accompanied with their simple instrumentation requirement and low production costs, make them attractive analytical tools suitable for a wide variety of applications. However analysis of very small concentration will result in insignificant changes in potentials which make the determinations prone to errors. A 10% activity change of a monovalent cation at 25 °C leads to a mere 2.4 mV change in the emf as predicted by Nernst equation. When the activity is doubled emf changes by 17.8 mV. Temperature changes and improper reference electrode themselves will give rise to an error of similar magnitude. Hence proper control of temperature, frequent recalibrations and use of highly reliable reference electrodes are mandatory for potentiometric analysis. These problems remain as stumbling blocks for the implementation of potentiometric sensors for applications as implantable electrodes and for remote sensing using wireless sensor networks. Hence researchers have come out with some modifications of the potentiometric analysis to overcome such obstacles. Some of the innovative modifications of potentiometric analysis attempted are high-amplitude sensing, backside calibration potentiometry, constant current coulometry and coulometric ion transfer [67]. These modifications are expected to extend the applications of potentiometric sensors to remote sensing and for the fabrication of implantable electrodes that can function for prolonged periods [67].

3. Voltammetric detection of phosphate

3.1. Voltammetric methods based on supramolecular recognition of phosphate

Designing of a good anion receptor requires selection of a proper signal unit and designing of an effective binding site in case of sensing of anions by voltammetry. Potentiometric determination does not require a signalling unit as it depends on the distribution of anions in the membrane containing the recognition molecule with the binding sites and the analyte solution and the resulting potential changes (Nernstian) at the interface. The factors to be considered while designing a selective receptor for anions are geometry, basicity of the anion and the nature of the solvent medium where sensing has to be carried out. Complementarity

Table 1
Analytical parameters for some potentiometric sensors for phosphate.

Sl. no.	ISE (active material)	Sensing parameters	Reference
1	Co electrode	Dynamic linear range 10^{-4} to 10^{-2} mol L^{-1}, with slopes ranging from −35 to −50 mV $decade^{-1}$	[40]
2	Co wire	Flow injection potentiometric (FIP) determinations of dihydrogenphosphate ($H_2PO_4^{2-}$) in fertilisers and waste waters at pH 5 have been carried out. Nernstian slope of −58.7 mV per decade was obtained in the concentration range 10^{-4} to 10^{-2} mol L^{-1}	[41]
3	Co micro electrode (10 μm)	Dynamic linear range 10^{-5} to 10^{-1} mol L^{-1} Detection limit 7.5×10^{-5} mol L^{-1} .	[42]
4	Oxidised cobalt metal electrodes	Linear dynamic range10^{-5} to 10^{-2} mol L^{-1} at pH 4.0; (log $KH_2PO_4^{-1}$ pot < −3).	[43]
5	Co wire electrode	-38.0 ± 0.5 mV $decade^{-1}$ in the range 5×10^{-3} to 10^{-5} mol L^{-1} at pH 5.0 Detection limit 10^{-6} mol L^{-1}	[44]
6	Bis (p-chlorobenzy1) tin dichloride	2.0×10^{-4} to 8.6 10^{-5} mol L^{-1} (This range is reported in the presence of different interfering anions)	[51]
7	Bis(terthiophene)-appended uranyl-salophen complex, comprising *N*,*N*′-bis[4-(5,2′:5′,2″-terthiophen-3′-yl)salicylidene]-1,2-ethanediamine–uranyl complexes (TUS), as a monomer for the electrochemical polymerisations (poly-TUS) on glassy carbon surfaces to form functionalised conducting polymer (CP) films	The CP/poly-TUS sensor showed a linear range between 1.0×10^{-1} and $1.0 \times 10^{-4.5}$ mol L^{-1} with a near-Nernstian behavior (−30.4 mV $decade^{-1}$) at a pH of 8.2. The detection limit of the electrode was $10^{-5.0}$ mol L^{-1} and the response time was <10 s	[61]
8	3-Decyl-1,5,8-triazacyclodecane-2,4-dione)	Linear dynamic range 10^{-6} to 10^{-1} mol L^{-1}	[62]
9	A zwitterionic bis(guanidinium) ionophore bearing an anionic *closo*-borane cluster	The lower detection limit for HPO_4^{2-} in an unbuffered solution is 8.7×10^{-8} mol L^{-1}	[64]

between the receptor and anion is highly crucial in determining selectivities. A useful way of grouping anion receptors is to consider the types of noncovalent interaction used to complex the anionic guest. These include electrostatic interactions, hydrogen bonding, hydrophobicity, coordination to a metal ion, and combinations of these interactions working together. Electrochemical molecular recognition is an expanding research area at the interface of electrochemistry and supramolecular chemistry [29,30]. Schematic diagrams of this approach are shown in Fig. 2. A variety of organic, organometallic, and inorganic redox active centres (signal units) have been incorporated into various molecular recognition frameworks containing the binding sites and have been shown to electrochemically detect anions. Electrochemical interrogation of phosphate anions by techniques such as cyclic voltammetry (CV) has been widely used in anion recognition for its own advantages like convenience in its operation, low cost and small sample volumes.

3.1.1. *Metallocene receptor systems*

3.1.1.1. Cobaltocene based receptors. The first redox-active class of anion receptors, based on the cobaltocenium moiety was reported by Beer and Keefe in 1989 [68]. Since then, a plethora of acyclic, macrocyclic, and calixarene receptors containing cobaltocenium (Cp_2Co) have been prepared. Cyclic voltammetric experiments demonstrated that all these receptors could electrochemically sense anions. The addition of anions to solutions of the receptors in acetonitrile resulted in significant shift of the reversible Cp_2Co^+/Cp_2Co redox couple towards lower potentials. The complexed anionic guest effectively stabilises the positively charged cobalt centre making it more difficult to reduce. For example, complexation of chloride ions by amide functionalised cobaltocenium receptors induced shifts between 30 mV and 85 mV, whereas larger magnitudes of 200 mV and 240 mV were observed for the complexation of dihydrogenphosphate towards lower potentials [69,70]. The anion-coordination properties of the cobaltocenium bridged calix[4]arene receptors are dependent upon the degree of preorganisation of the upper rim. Recognition can be tuned in favour of phosphate anions by exchanging the positions of the tosyl substituent on the lower rim of the calix[4]arene which had a dramatic influence on the anion coordination properties of the upper rim [71–73].

3.1.1.2. Ferrocene based receptors. In the electrochemical anion recognition area, especially with respect to phosphate anions, ferrocene group has proved to be a very good signal unit because of its stable electrochemical properties and its ease of detection by methods such as CV. Besides, the ferrocene group can modulate the binding event through alternating between its two redox states. As for binding site, amide [74], urea [75], and the hydroxyl group [76] that can form hydrogen bonds with anions are commonly used.

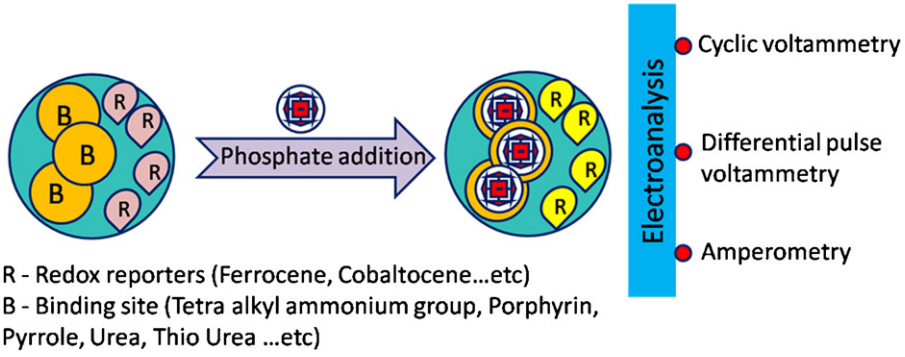

Fig. 2. Recognition of phosphate based on supramolecular interactions.

Quarternised nitrogen [77] and positively charged pyridine [78] are also chosen as binding sites on the basis of the electrostatic interaction. Others that can provide shape complementation [79] are also considered. Ferrocenyl esters such as glycidyl ester of ferrocene carboxylate (GEFC) and 1,3-diferrocenecarboxylic acid diacylglycerol (DFCDG), 1,1′-*N*,*N*′-ferrocenoyl bisamino acid methyl esters and bisferrocenyl-substituted urea and thiourea and trisferrocenyl-substituted guanidine derivatives were also evaluated for sensing anions [80–82]. However most of the receptors respond not only to phosphate but also to anions like bisulphate, fluoride anions etc., Hence sensitivity and selectivity are still subjects of further investigations. It is now well accepted that one single interaction in the receptor molecule such as hydrogen bond or electrostatic interaction or shape complementation may not be enough to improve the selectivity and sensitivity for phosphate, especially in an aqueous environment [83]. Thus, combination of several interactions is being considered. It is believed that multiple binding sites involving several different binding groups such as those that can form hydrogen bond or that can provide electrostatic interactions, will improve the sensitivity and selectivity of anion receptors. A new ferrocenyl anion receptor with specially designed multiple binding sites viz., amide and positively charged nitrogen (*N*,*N*,*N*,*N*-(dimethyl, ethyl, ferrocenecarboxylic amidodimethylene) ammonium fluoborate) was synthesised by Tan et al. [84]. Compared to its counterpart with just a single binding site of amide, this compound with multiple binding sites showed higher sensitivity to $H_2PO_4^-$ and thus proved the enhancement effect of the multiple binding sites [84]. Ferrocene substituted calix(4)pyrroles have been synthesised and investigated using acetonitrile:DMSO mixture (9:1) by cyclic voltammetry and square wave voltammetry and it was found to bind fluoride, chloride and dihydrogenphosphate anions [85]. A neutral redox-active receptor (ferrocene functionalised calix[4]pyrrole) was used as an active component in carbon paste electrodes as ISEs, for the detection of anions in aqueous solution. Measurements with carbon paste electrodes were conducted using Osteryoung square-wave voltammetry. Amongst the anions studied, dihydrogenphosphate and fluoride caused the strongest decrease of peak current (approximately 25%), followed by bromide and chloride [86]. The electropolymerisation of a simple monoamidoferrocene derivative containing a pyrrole group is a straightforward way to synthesize redox polymer films that can sense $H_2PO_4^-$, ATP^{2-} and HSO_4^-, with excellent selectivity for the former anions [87]. Platinum and gold microelectrode arrays (MEAs), fabricated on silicon substrates with different geometric characteristics, were surface-modified by the potentiostatic electropolymerisation of the pyrrole-ferrocene derivative, in the case of the platinum MEAs, and by the chemisorption of the thiol-functionalised ferrocene, in the case of the gold MEAs. The modified MEAs were investigated for the detection of the dihydrogenphosphate mono-anion in nonaqueous media via differential pulse voltammetry. This was based on electrostatic interactions and/or hydrogen bonding between the target anion and the amide-ferrocene or ammonium-ferrocene functionalised electrode surfaces. A decrease in the ferrocene (Fc) oxidation peak current with a concomitant increase in the peak current of a new peak at lower potentials was observed when the concentration of the dihydrogenphosphate was increased. This method exhibited very good selectivity for $H_2PO_4^-$ anions compared to ATP, HSO_4^- and NO_3^- ions and the analysis was performed in nonaqueous solution using differential pulse voltammetry [88]. Pentamethyl amidoferrocene dendrimers and silane based ferrocene dendrimers (in solution and in the modified phase) have also been evaluated for the recognition of anions like phosphate, ATP, HSO_4^- etc. [89,90]. Amide substituted tetrathiafulvalene derivatives are used with some success for the selective determination of $H_2PO_4^-$ over other anions in nonaqueous medium [91]. The main disadvantage of all these systems is the lack of selectivity in the majority of the cases and the studies are confined only to aprotic media with one or two exceptions [86,87,92]. The reference [87] discusses selective detection of phosphate in aqueous environment whereas the references [86] and [92] discuss selective sensing of phosphate in non-aqueous conditions.

3.1.2. Anion complexation through second-sphere coordination

3.1.2.1. Transition-metal bipyridyl based receptors. The use of a second coordination sphere of metal complexes as a basis of anion recognition is another method for the development of hydrogen-bond based inorganic anion receptor. The redox-active and photoactive ruthenium(II) bipyridyl moiety, in combination with secondary amide groups, incorporated into acyclic, macrocyclic, and lower-rim calix[4]arene structural frameworks are shown to produce a new class of anion receptors capable of optical and electrochemical sensing [93]. Single-crystal X-ray structures of the $H_2PO_4^-$ complex of the Ru(II) bipyridyl compound in combination with secondary amide groups incorporated into calixarene frame work highlight the importance of hydrogen bonding to the overall second sphere anion complexation process. Three hydrogen bonds (two amide and one calix[4]arene hydroxyl) stabilise the $H_2PO_4^-$ anion. The ruthenium ion is dipositive, and hence, electrostatic interactions are particularly favourable. The macrocyclic receptors form highly selective and thermodynamically stable complexes with $H_2PO_4^-$. Electrochemical anion recognition experiments showed substantial anion-induced cathodic perturbation of the phosphate complex in agreement with stability constant values of 28 000 M^{-1} for $H_2PO_4^-$ in DMSO, capable of selectively sensing $H_2PO_4^-$ in the presence of 10-fold excess amounts of HSO_4^- and Cl^-.

3.1.3. Heteroditopic sensing

The design of heteroditopic ligands that contain two quite different binding sites for the simultaneous complexation of cationic and anionic guest species is an emerging field of supramolecular chemistry. These multisite ligands are able to bind a single heteroditopic guest or simultaneously bind two non-identical guests. The invention of convergent heteroditopic hosts is a challenging problem in molecular design because the binding sites have to be incorporated into a suitably preorganised scaffold that holds them in close proximity, but not so close that sites interact. Ferrocene-based ionophores substituted with crown ethers or polyaza-macrocycles exhibit interesting electrochemical cation recognition effects because the complexing ability of the ligand can be switched on and off by varying the applied electrochemical potential. Owing to the relatively strong hydrogen bonding ability of the urea group, a number of molecules possessing the urea motif have been designed as neutral receptors for various anions. By combining the redox activity of the ferrocene moiety with the anion binding ability of the urea group and a crown ether moiety as an alkaline metal-binding site, a new heteroditopic ferrocene based ligand capable of the simultaneous binding of anions and cations can be designed. A heteroditopic ligand containing urea and a crown ether group synthesised by Otón et al. was studied by CV in dichloromethane containing 0.1 M $TBAClO_4$ as supporting electrolyte .The compound exhibited a reversible one-electron oxidation process corresponding to ferrocenium–ferrocene (Fc^+/Fc) couple. Electrochemical anion and cation sensing experiments were carried out by differential pulse voltammetry (DPV). On stepwise addition of 1.5 equivalents of F^- (as its TBA^+ salt) led to a modest cathodic shift of −52 mV. However, upon addition of 2 equivalents of $H_2PO_4^-$ a very large shift of 190 mV occurred in the negative direction, reflecting a strong binding of the guest upon oxidation of the ferrocene unit. Maximum perturbation of the differential pulse voltammetry (DPV) output was obtained with 2 equivalents of added $H_2PO_4^-$ anion. Remarkably, the presence

of Cl^-, Br^-, HSO_4^- and NO_3^- anions had no effect on the DPV, even when present in large excess [94]. Guo et al. synthesised a ferrocene-based 1,3-alternate thiacalix[4]arene ditopic receptor that contained four identical polyether arms terminated with the ferrocene amide moieties. Cyclic voltammetric studies conducted in a nonaqueous medium containing 1:1 dichloromethane and acetonitrile have revealed that this redox-active receptor can be used as an electrochemical sensor to recognise both europium (Eu^{3+}) and dihydrogenphosphate $H_2PO_4^-$ ions with a high selectivity [92]. Beer et al. demonstrated using water soluble pH dependent polyazaferrocene macrocyclic ligands that the pH dependent electrochemical recognition of transition metal cations and phosphate anions in the aqueous environment. At low pH, the compound exists in the protonated form and can be used to determine biologically important anions like phosphate and ATP in the aqueous environment. At high pH they exhibit recognition properties towards cations especially with respect to Cu^{2+} ions [95].

3.2. Voltammetry at the interface between two immiscible electrolyte interafces (ITIES)

Many calixarene compounds are known to be anion-selective towards halides (Cl^-, Br^-, I^-) [96–98]. Of late the use of modified calix[4]arene and calix[6]arene molecules have effectively been used for phosphate sensing in PVC-membrane ISEs [99,100]. The urea functionalised calixarene was demonstrated to exhibit selectivity towards phosphate compared to common anions like sulphate and chloride. However anions like nitrate and perchlorate interfered in the detection [36]. The disadvantages associated with interference using ISEs may be overcome by the introduction of a supplementary measurement dimension. The ions that cannot be distinguished under equilibrium potentiometry conditions can be analysed with the help of voltammetric or amperometric techniques at the liquid–liquid interface which allows the separation of co-transferring ions on the potential axis. This leads to an improvement in the performance of the sensing process. The interaction of a urea-functionalised calix[4]arene ionophore and phosphate was investigated by voltammetric ion transfer at the interface between two immiscible electrolyte solutions (ITIES). Voltammetry at the ITIES established that the ionophore-facilitated transfer of monohydrogenphosphate occurred in preference to dihydrogenphosphate transfer. The results are comparable with previously reported data on the potentiometric evaluation of this calixarene as an ionophore in PVC-membrane electrodes [101]. The data provide the foundation for the development of amperometric monohydrogenphosphate sensors based on the ion-transfer principle

3.3. Thermal modulation (TM) voltammetry

Thermal modulation voltammetry involves the determination of changes in the voltammetric signals ($\Delta I/\Delta V$), when the electrode/electrolyte surface is periodically heated using laser sources. The thermal modulation of the interface changes the standard entropy of the electrode reaction. The temperature coefficient of the standard potential, $(\partial E^\circ/\partial T)$, for the electrode reaction of $Ox + ne \Leftrightarrow Red$ is equal to the product of the number of electrons, the Faraday constant and the standard entropy change, $-nF\Delta S^\circ$. Subsequently, when the temperature is increased from T to $T + \Delta T$, and if the electrode reaction has a positive value of the standard entropy change, the standard potential shifts to a negative direction, and vice versa. Since the limiting current increases with the diffusion coefficient, and hence with the temperature, the limiting current at $T + \Delta T$ is always greater than that at T. Such temperature effects on thermodynamics and diffusion lead to a minor difference between two voltammograms at T and $T + \Delta T$, where ΔT is much less than T. TM voltammetry is a sensitive enough to detect such small differences. The measurement involves a periodic heating and lock-in detection system in addition to the conventional voltammetric instruments. TM voltammetry has been explored for the detection of phosphate in natural water samples by using a He—Cd dual laser as a heating source and a graphite-reinforced carbon (GRC) electrode. The heteropoly ion, i.e., 12-molybdophosphate ion ($[PMo(VI)_{12}O_{40}]^{3-}$), was formed through a reaction between phosphate and molybdate ions in an acidic solution, and its electroreduction was investigated in a flow electrolytic cell by TM voltammetry [102]. Measured TM voltammograms showed two peaks corresponding to two successive two-electron reductions of the 12-molybdophosphate ion, and the peak intensities were proportional to the concentration of the phosphate ion. Because of the strong adsorption of 12-molybdophosphate ion onto the graphite reinforced carbon electrode, a detection limit as low as 0.8×10^{-9} mol L^{-1} was achieved. The determination of phosphate ion in river water was carried out by TM voltammetry. The results obtained were similar to those obtained by the spectrophotometric molybdenum blue method. These results prove the significance of TM voltammetry as an electroanalytical method for the determination of phosphate [102].

4. Amperometric detection of phosphate

4.1. Amperometric detection via electrochemical reduction of phosphomolybdates

The popular analytical method for the determination of phosphate involves treatment of the sample with an acidic molybdate solution to convert the phosphate into the Keggin anions ($PMo_{12}O_{40}{}^{3-}$) and subsequent chemical reduction leading to mixed molybdenum oxidation state [103]. These ions are intensely blue coloured which allows spectrophotometric determination of trace level phosphate in an analyte. As a routine analytical method, this chemistry is carried out in an automated continuous flow assembly. The chemistry of these Keggin anions is complex and the rate of formation, stability and ratio of isomers depend strongly on the solution conditions, including pH, and the method suffers from interferences from silicate, arsenate etc. Sometimes addition of organic solvents is required for the selective extraction of the desired analyte. Even the order of addition of reagents affects the results of the experiment and therefore the method is not entirely and universally satisfactory. To avoid the complications arising from the use of chemical reducing agents, the Keggin anions are reduced electrochemically followed by spectrophotometric analysis. This technique has successfully been employed for the determination of orthophosphates in beverages, waste waters and urine samples [103]. FIA (flow injection analysis) and spectrophotometric techniques, commonly used for the measurement of phosphate in the laboratory, are not suitable for on-site testing and monitoring. The use of direct eletroreduction techniques offers portability and excellent sensitivity which make them very attractive for on site monitoring of phosphate. Furthermore the interference from ions like silicate and arsenate can be completely avoided as reductions of their respective species occur at different potentials. The potential dependent selectivity combined with the portability and prospect of miniaturisation make the electrochemical determination of phosphate using molybdophopshate highly versatile. Several reports on the electrochemical determination of phosphate using phosphomolybdates are available in the literature [5,104–110]. Amperometric detection in flow injection analysis is widely reported in the literature [5,107–110]. One of the papers describes formation of phosphomolybdate complex in presence of nitric acid, ammonium molybdate and phosphate and its subsequent reduction at a carbon paste electrode, polarised at +0.3 V (vs

Ag/AgCl) [5]. The major characteristics of the method were simplicity of the equipment, limited consumption of reagents and low limit of detection (0.3×10^{-6} mol L−1) with a linear range between 1 and 20×10^{-6} mol L^{-1}. The interference of silicate was completely eliminated by using appropriate concentrations of nitric acid and ammonium molybdate. This method was successfully applied to orthophosphate analysis in cyanobacterial biofilms collected from Roman catacombs [5]. The potential dependent selective determination of silicates and phosphates was also evaluated by carrying out the voltammetry of the molybdosilicate and molybdophosphate complexes, formed by the addition of hexafluorosilicate and phosphate to an acidic sodium molybdate solution, at gold microdisk electrodes [109]. It is shown that the reaction conditions influence both the kinetics of formation of the complexes and their voltammetry. It is possible to find the conditions where the steady state amperometric response of the Au microdisk electrodes allows a rapid and convenient method for the determination of silicate and phosphate at concentrations in the range $1–1000 \times 10^{-6}$ mol L^{-1} [111]. However from the perspective of researchers involved in remote sensing, it has been reported that the colorimetric analysis of phosphate based on ammonium molybdate meets the stringent analytical requirements needed for remote sensing. The power requirement for the colorimetric detector is also suitable for remote sensing. Similar ruggedness can also be developed in the case of electrochemical sensing of phosphate using ammonium molybdate [112]. The reaction occurring between molybdate ions and phosphate ions resulting in blue colour is as follows:

$$7PO_4^{3-} + 12Mo_7O_{24}^{6-} + 72H^+ \rightarrow 7PMo_{12}O_{40}^{3-} + 36H_2O \quad (4)$$

$$PMo_{12}O_{40}^{3-} + 2nH^+ + 2ne \rightleftharpoons [H_{2n}PMo_{12}O_{40}]^{3-} \quad (5)$$

A perovskite-type oxide-based electrode showed good properties of amperometric sensing to hydrogen-phosphate ion. The carbon electrode loaded with $La_{0.9}Ce_{0.1}CoO_3$ showed remarkable selectivity to HPO_4^{2-} among the examined anions like F^-, Cl^-, Br^-, SCN^-, NO_3^-, SO_4^{2-}, CO_3^{2-} and ClO_4^-, although it received serious interference from I^-. The $LaCoO_3$ thin film sensor device responded to HPO_4^{2-} at concentrations between 1.0×10^{-6} and 1.0×10^{-1} mol L^{-1} [113].

4.2. Indirect determination of phosphate

A highly selective enzymeless approach using a $Ni(OH)_2$/NiO(OH) modified barrel plated nickel electrode (Ni-BPE) in alkaline media for the determination of phosphate (PO_4^{3-}) by flow injection analysis (FIA) has been reported recently [114]. The presence of $Ni(OH)_2$/NiOOH activates the adsorption of phosphate at the electrode surface which inhibits the current corresponding to the electrocatalytic oxidation of glucose in 0.1 M NaOH solution. Under the optimised conditions of flow rate (300 μL min^{-1}), detection potential (0.55 V vs Ag/AgCl) and with 25×10^{-6} mol L^{-1} glucose in 0.1 M NaOH as carrier solution, the calibration curve showed a linear range up to 1×10^{-3} mol L^{-1}. Probable interference from coexisting ions was also examined. The results confirmed that the sensor could be used for the determination of phosphate in the presence of nitrate, chloride, sulphate, acetate, oxalate, carbonate. It could also be used in the presence of other anionic species of toxicological and environmental interest, such as chlorate, chromate, and arsenate ions. The electrode could be efficiently regenerated without further treatment under the hydrodynamic condition. For eight continuous injections of 40×10^{-6} mol L^{-1} PO_4^{3-}, a relative standard deviation of 0.28% was obtained, indicating good reproducibility of the proposed method. A detection limit of 0.3×10^{-6} mol L^{-1} was achieved by this method. A schematic diagram of the sensing mechanism is given in Fig. 3.

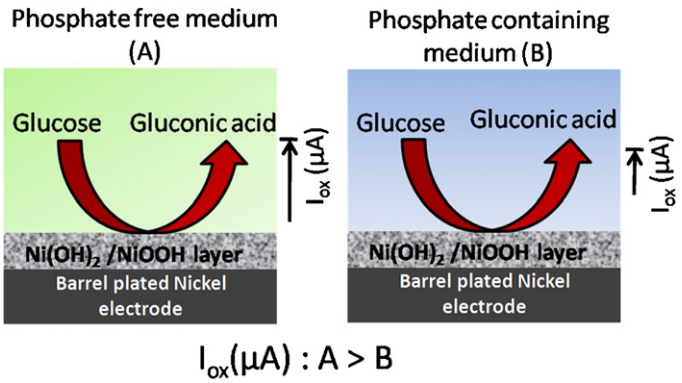

Fig. 3. Schematic diagram of phosphate sensing based on the inhibition of the current due to the oxidation of glucose on $Ni(OH)_2$/NiOOH electrode in presence of phosphate ions.

4.3. Amperometry coupled with ion chromatography

An amperometric sensor intended especially for non-electroactive ions, functioning under flow injection mode, was applied as a novel detector in suppressed ion chromatography. It consists of a carbon paste electrode modified with either a polycationic or a polyanionic polymer holding a suitable charge transfer mediator ($[Fe(CN)_6]^{3-}$ or Cu^{2+}), functioning in an indirect amperometric mode. The detection mechanism involves ion exchange between the non-redox ionic analyte and the electroactive mediator, in the polymer particles positioned at the electrode surface, followed by the electrochemical transformation of the mediator species leached out of the polymer at the electrode/solution interface. The estimation was accomplished in the absence of added supporting electrolyte. Optimisation was performed to get the highest faradic signals, by varying a range of experimental parameters (i.e. applied potential, composition of the electrode). These systems were then successfully applied to the analysis of mixtures of cations (Li^+, Na^+, K^+, NH_4^+, Ca^{2+}, Mg^{2+}) and anions (F^-, Cl–, NO_2^-,NO_3^-, SO_4^{2-}, PO_4^{3-}) following chromatographic separation. Good operational stability was observed, with typically less than 5% signal loss for 50 consecutive measurements [115].

5. Sensing of phosphate using self- assembled monolayers

Self-assembled monolayers (SAMs) of thiol-derivatised molecules on gold substrates have recently received substantial consideration in connection with their potential applications for sophisticated designs of molecular-based electronics, chemical sensors, and nanopatterning. Reports on SAM based platforms for phosphate sensing is available in the literature [116,117]. In one of the reports, ferrocene based thiols, appended with binding site for oxoanions (most often amide groups or trialkyl ammonium groups) are self-assembled on gold electrodes and the sensing of phosphate anions are evaluated in organic and inorganic media using voltammetric shifts produced for the ferrocene/ferrocenium couple as a result of binding the anions [116,117]. However this method responded to several anions and is not selective.

In another case the blocking of electron transfer properties for ferrocyanide electron transfer across a SAM modified electrode containing a binding site for anions (porphyrin or zirconium(IV) ions) was evaluated for the sensing of phosphate anions. This approach was found to be selective for phosphate over many other anions [118,119].

A SAM based platform derived from mercaptopropionic acid was further functionalised with Zr(IV) ions and was found to be an effective modified electrode for the sensing of phosphate anions. The

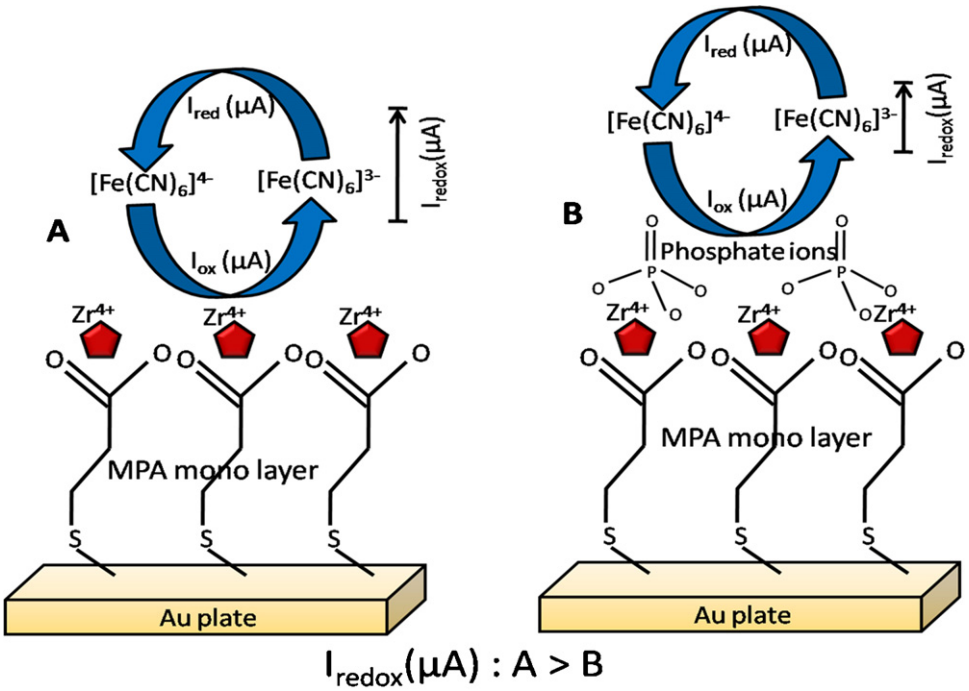

Fig. 4. Schematic diagram of phosphate sensing based on the blocking of the ferrocyanide electron transfer kinetics when phosphate ions interact with Zr(IV) ions linked to a self-assembled monolayer of mercaptopropionic acid.

method and applicability of the sensor were successfully tested by detection of phosphate in blood serum after deproteinisation of the sample without interference from the sample matrix [119].

A schematic diagram of the sensing mechanism is given in Fig. 4.

6. Unconventional methods for detection of phosphate

The detection of phosphate ions by using certain unconventional methods has also been reported in the literature. Some of these are, (a) following the intensity of electrochemiluminescence changes of the complex formed between molybdophosphates and a luminescent dye [34]. For example, the electrochemiluminescence (ECL) method was carried out with a hydrophobic ion dissociated complex formed between molybdophosphoric heteropolyacid and protonated butylrhodamine B (BRhB). The complex was selectively extracted into the bulk of paraffin oil based carbon paste electrode. The (ECL) was followed at +1.3 V in alkaline medium. Under the optimum experimental conditions, the ECL intensity was linear with the concentration of phosphate (as phosphorus) in the range of 6.4×10^{-9} to 1.0×10^{-7} mol L^{-1}. The detection limit was 2.5×10^{-12} mol L^{-1}. The proposed method has been applied successfully to the analysis of phosphate in the water samples [34]. (b) Following the reduction in transmittances of cobalt oxide thin film electrodes induced by the adsorption of phosphate at fixed potential [35]. (c) Following the mass changes associated with the inclusion of phosphate anions in conducting polymer films like PEDOT(polyethylenedioxythiophene) using a quartz crystal microbalance, QCM [120]. (d) A microfluidics-based ion-channel sensing system for nonelectroactive anions under negative separation electric field that relies on amperometric response of the oxidation of a carbon fiber electrode [121].

7. Bioelectroanalytical detection of phosphate

Biosensors for the determination of phosphate are normally based on mono- or multi-enzymatic reactions where phosphate acts as inhibitor or substrate. The enzyme-based amperometric biosensors are highly advantageous due to the high selectivity of the biorecognition element and the sensitivity of the electrochemical signal transduction. Despite the promise of high selectivity, phosphate selective enzymes are not readily amenable to direct electrochemical interrogation. One of the enzymes often employed for the sensing of phosphate is pyruvate oxidase (POD). The reaction involves the conversion of pyruvate to acetyl phosphate in the presence of oxygen to yield hydrogen peroxide. The concentration of phosphate is inferred from the amperometric measurement of either oxygen depletion or the increase in concentration of hydrogen peroxide [4,20,122–124]. These detection modes have a number of limitations. Measurement of a stable and clear-cut signal for the reduction of oxygen is often difficult and will be tricky in situations where the concentration of oxygen is very low. The oxidation of peroxide is hampered by poor electrode kinetics at conventional electrode materials and requires a large over potential for oxidation to give rise to a quantifiable current signal and hence prone to interferences from oxidisable impurities in the solution. In certain cases where oxygen concentration is a limiting factor, mediators are employed for the detection of phosphate. Osmium bipyridyl units functionalised pyrrole monomer, copolymerised electrochemically with thiophene was used as a mediator for pyruvate oxidase electron transfer and the detection of phosphate was demonstrated successfully using this mediator [124]. The sensitivity of detection achieved in this case is 0.2 A cm^{-2} and detection range is between 0.02 mol L^{-1} and 5 mol L^{-1}. The POD catalysed enzymatic reaction with phosphate as the substrate is as follows:

$$\text{Pyruvate} + \text{phosphate} + O_2 \xrightarrow{(\text{POD})} \text{Acetyl phosphate} + CO_2 + H_2O_2 \quad (6)$$

Nucleoside phosphorylase has been used [125,126] for the sensing of inorganic orthophosphate based on the following reactions

$$\text{Inosine} + \text{orthophosphate} \xrightarrow{(\text{Nucleoside phosphorylase})} \text{Ribose-1-phosphate} + \text{Hypoxanthine} \quad (7)$$

$$\text{Hypoxanthine} + 2H_2O + 2O_2 \rightarrow \text{Uric acid} + 2H_2O_2 \quad (8)$$

The concentration of phosphate was followed by studying the consumption of oxygen caused by the reaction with hypoxanthine which is generated during the enzymatic reaction between inosine and orthophosphate. The enzyme was immobilised on a membrane prepared from cellulose triacetate and was fixed on top of a Clark-type oxygen electrode. A detection limit of 10^{-4} mol L^{-1} phosphate was achieved by this method. Later d'Urso and Coulet [127] and Haemmerli et al. [128] were able to increase the sensitivity of this method by using a hydrogen peroxide transducer instead of using an oxygen electrode and a detection limit of 10^{-7} mol L^{-1} was confirmed. The ability to make use of the uric acid signal provides an important operational advantage. Urate is endogenous to physiological systems and hence would prove to be a substantial interferent in actual analysis of clinical samples [23]. In the context of environmental analysis, it is likely that only certain samples would be expected to contain the purine and hence the direct oxidation of the base at the electrode can be assumed to be derived solely from the enzymatic sensor assembly. The advantage of exploiting this label rather than peroxide lies in the relatively low oxidation potential of the purine (~+0.2 to +0.5 V). The oxidation of peroxide is associated with poor electrode kinetics and large over potentials (~+0.8 to +1 V vs Ag/AgCl) at conventional electrode substrates.

An enzymatic sensor based on four different enzymes for phosphate detection was reported using maltose phosphorylase (MP), acid phosphatase (AP), glucose oxidase (GOD) and mutarotase (MR) with the following reaction sequence [129].

$$\text{Maltose} + \text{phosphate} \xrightarrow{\text{MP}} \beta\text{-D-glucose-1-phosphate} + \alpha\text{-D-glucose} \quad (9)$$

$$\beta\text{-D-glucose-1-phosphate} \xrightarrow{\text{AP}} \beta\text{-D-glucose} + \text{phosphate} \quad (10)$$

$$\alpha\text{-D-glucose} \xrightarrow{\text{MR}} \beta\text{-D-glucose} \quad (11)$$

$$\beta\text{-D-glucose} + O_2 \xrightarrow{\text{GOD}} \beta\text{-D-gluconic acid} + H_2O_2 \quad (12)$$

$$H_2O_2 \rightarrow 2H^+ + 2e + O_2 \quad (13)$$

The combination of the former two enzymes generates two glucose molecules per reaction cycle and recycles one molecule of phosphate, and the oxidation of glucose is catalysed by the glucose oxidase enzyme after its mutarotation. The formation of hydrogen peroxide during the enzymatic reaction can be monitored electrochemically at a platinum electrode. Based on this study, both tri- and bi-enzymatic sensors for phosphate detection have been described. Mousty et al. [19] used a simple method to fabricate an amperometric phosphate biosensor containing MP, MR, and GOD with a linear range of $1–50 \times 10^{-6}$ mol L^{-1}. Hüwel et al. [130] successfully applied MP and GOD as the bioelements of a simple bi-enzymatic sensor, in which the first enzyme consumed phosphate as a cosubstrate and yielded a product that was a substrate for the second enzyme. However, more enzymes involved in the sensor system lead to more non-specific response due to the presence of substrates for the next enzymes. Furthermore, the instability of each enzyme caused fluctuations in the sensor performances. For example, in the phosphate detection system consisting of nucleoside phosphorylase and xanthine oxidase, the degradation of inosine often restricted the dependability of phosphate analysis [131]. For this bi-enzyme system, no linear range was found even though it sensed phosphate [131,132]. Further immobilisation of three to four enzymes in spatially separated planes is a challenging task. Utmost care should be taken to keep the enzymes in the active state in the immobilised conditions in the presence of other enzymes. When more enzymes are present, the system becomes complicated and the results will be difficult to understand and troubleshooting will be hardly practicable. Fouling at the electrode surface will be another issue to be addressed in this case. Hence, multi-enzyme systems are rather complicated and expensive.

Based on a monoenzymatic reaction, Zhang et al. [133] developed a conductometric biosensor which measures the conductance changes associated with the following reaction on the addition of phosphate. The detection limit achieved was 1×10^{-6} mol L^{-1}. No interference from other anionic species was detected. The conductometric biosensor exhibited a long-term storage and operational stability as well as a good thermal stability. Measurements in the real water samples were satisfactory. The enzymatic reaction is as follows;

$$\text{Maltose} + \text{phosphate} \xrightarrow{\text{MP}} \beta\text{-D-glucose-1-phosphate} + \alpha\text{-D-glucose} \quad (14)$$

Another enzyme that is often used for the determination of phosphate esters is the enzyme alkaline phosphatase (ALP) and it is mainly used for the determination of phosphate esters or for the determination of the enzyme activity. Hence the direct determination of inorganic phosphate is not possible with this enzyme. The adaptation of the methodology for the detection of orthophosphate relies upon the inhibitory action of the latter on the hydrolysis of the ester substrate. The sample is assayed using known concentrations of enzyme/substrate with the decrease of the signal from that expected in the absence of added phosphate being inversely related to the concentration of the latter. Amino phenol phosphates are generally used as the substrate for alkaline phosphatase enzyme. However the aminophenol that is generated during the enzyme hydrolysis gets oxidised only at high overpotential and this method suffers from interferences. Second issue that arises is that the electrochemical oxidation leads to polymeric deposits on the electrode. These tend to block the electrode reducing sensitivity and compromising the reproducibility of the technique. Hence phosphate esters containing ferrocene or aminophenyl derivatives that can be oxidised at low overpotentials were synthesised and explored for phosphate detection. The alkaline phosphatase catalysed enzymatic reaction using *p*-aminophenyl phosphate as the substrate is as follows:

$$p\text{-aminophenyl phosphate} \xrightarrow{\text{ALP}} p\text{-aminophenol} \quad (15)$$

$$p\text{-aminophenol} \rightarrow \text{Quinone imine} + 2e + 2H^+ \quad (16)$$

The majority of systems using ALP make use of a combination of enzymes in order to produce an electrochemical label with facile electron transfer kinetics. Such systems rely upon the synergistic interaction of the multi-enzyme assembly to yield a product (typically peroxide) that is more amenable to electrochemical detection than the labelled esters. A typical bienzyme system reported in the literature involved ALP/GOD assemblies with glucose-6-phosphate as the key substrate in the reaction and the electrochemical label here is the oxygen that is consumed during the oxidation of glucose by glucose oxidase. Increased phosphate concentrations inhibit the production of glucose and hence the consumption of oxygen is decreased [134–136] as is the yield of peroxide [137]. The multi enzyme assembly thus constructed was found to work satisfactorily within a range of environmental matrices like fresh and sea water samples. Interference from heavy metal ions (mercuric, cupric and zinc) can occur, but these are not likely to appear in any appreciable concentration in natural samples. The limit of detection for phosphate using the ALP/GOX combination was typically 0.4 ppm (4 μM) and is comparable to those obtained using the molybdate

Table 2
Analytical parameters for some enzymatic sensors for phosphate.

Sl. no.	Enzyme electrode	Basis of measurement	Sensing parameters	Reference
1	Immobilising pyruvate oxidase (PyOD) on a screen-printed electrode	The enzymatic generation of hydrogen peroxide (H_2O_2) detected at +420 mV vs Ag/AgCl	Response time <2 s short recovery time (2 min). The time required for one measurement using this phosphate biosensor was 4 min, which was faster than the time required using a commercial phosphate testing kit (10 min). The sensor has a linear range from 7.5 to 6.25 × 10^{-6} mol L^{-1} phosphate with a detection limit of 3.6 × 10^{-6} mol L^{-1} Human salivary samples have been analysed for the phosphate content	[4]
2	Poly(carbamoylsulphonate) (PCS) hydrogel immobilised pyruvate oxidase	Enzymatically generated H_2O_2 monitored at +300 mV vs phthalocyanin/carbon (PC) reference electrode	Rapid phosphate process control monitoring in an experimental sequencing batch reactor (SBR) system. The signal response time was 1 min with a detection limit of 5 × 10^{-3} mol L^{-1}	[20]
3	Pyruvate oxidase immobilised on a copolymer formed electrochemically with $Os(bipy)_2pyCl^-$ modified pyrrole monomer of and thiophene on a platinum black	Detection of enzymatically generated H_2O_2 (+0.40 V vs Ag/AgCl)	Phosphate was measured similarly between 0.02 × 10^{-3} and 0.5 × 10^{-3} mol L^{-1} in the presence of pyruvate as co-substrate. The sensitivity of the sensor dropped to about 12% after 10 days	[122]
4	Covalent immobilisation of pyruvate oxidase (PyO) onto the nano-particle comprised poly-5,2′:5′,2″-terthiophene-3′-carboxylic acid, poly-TTCA (nano-CP) layers on a glassy carbon electrode	Detection of enzymatically generated H_2O_2 (+0.40 V vs Ag/AgCl) in a phosphate solution.	Dynamic linear range 1.0 × 10^{-6} mol L^{-1} to 100 × 10^{-6} mol L^{-1} and the detection limit was determined to be about 0.3 × 10^{-6} mol L^{-1} . The response time of the biosensors was about 6 s	[123]
5	Immobilisation of pyruvate oxidase (PyO_x) on a polyion complex membrane	Detection of enzymatically generated H_2O_2	Detection limit 0.2 × 10^{-6} mol L^{-1} of phosphoric acid	[124]
6	Maltose phosphorylase, acid phosphatase, glucose oxidase and mutarotase were coimmobilised on a regenerated cellulose membrane which was mounted on the tip of a platinum	A mperometric electrode for the detection of enzymatically formed hydrogen peroxide	Detection limit of 10^{-8} mol L^{-1} was obtained Dynamic range 0.1–l × 10^{-6} mol L^{-1}, Relevant for the monitoring of water pollution	[129]
7	Maltose phosphorylase (MP) from recombinant *Escherichia coli* immobilised on a planar interdigitated electrode by cross-linking with saturated glutaraldehyde (GA) vapour in the presence of bovine serum albumin (BSA)	Conductometric biosensor	Temperature stability 20–50 °C Response time 10 s. The sensor has two linear ranges, one is from 1.0 to 20 × 10^{-6} mol L^{-1} phosphate with a detection limit of 1.0 × 10^{-6} mol L^{-1}, and the other is between 20 and 400 × 10^{-6} mol L^{-1} phosphate. Storage life in citrate buffer was two months. No obvious interference from other anionic species like SO_4^{2-}, Cl^-, NO_3^-, NO_2^- and HCO_3^- was detected. The biosensor is suitable for use in real water samples	[133]
8	A potato (*Solanum fuberosum*) tissue slice immobilised glucose oxidase coupled with a Clark oxygen electrode	Measurement is based on the inhibition by phosphate of potato acid phosphatase catalysed glucose and phosphate	Lower detection limit 2.5 × 10^{-5} mol L^{-1}; Sensor is stable for 28 days or 300 assays	[134]
9	The co-immobilisation of polyphenol oxidase and Alkaline phosphatase leads to a bienzyme electrode for the determination of phosphate based on the inhibition effect of hydrolysis by phosphate	Sensing is based on the detection of the enzymically generated *o*-quinone at −0.2 V	The sensitivity and detection limit of the biosensor for phosphate were 1.27 mA M^{-1} cm^{-2} and 2 × 10^{-6} mol L^{-1}	[139]
10	Phosphate-binding protein (PBP) from *Escherichia coli*. PBP was immobilised on a sheet of nitrocellulose membrane by cross-linking and the membrane potential of the immobilised PBP was measured		The response was selective to phosphate among other anions. Under optimum conditions 0.1–1.5 × 10^{-3} mol L^{-1} phosphate can be determined with this system	[141]

systems. The alkaline phosphatase catalysed enzymatic reaction with glucose-6-phosphate as the substrate is as follows:

$$\text{Glucose-6-phosphate} \xrightarrow{\text{ALP}} \text{Glucose} + H_3PO_4 \quad (17)$$

$$\text{Glucose} + O_2 \rightarrow \text{Gluconic acid} + H_2O_2 \quad (18)$$

Improvements in detection limit can be achieved through the catalytic cycling of the hydrolysed label. Hydroquinone monophosphate was used as the substrate for alkaline phosphatase. The hydrolysis product, hydroquinone, is capable of fast redox interconversion at the electrode surface. When ALP is combined with the glucose oxidase enzyme, in presence of an excess of glucose (to keep the FAD groups of the enzyme in the reduced state), the electrochemical oxidation product (benzoquinone) of the hydrolysis product hydroquinone, chemically reacts with the glucose oxidase and gets converted back to hydroquinone which can get oxidised at the electrode surface in a facile manner. A catalytic cycle builds up through which the current recorded at the electrode is effectively amplified. This method leads to subpicomolar detection of ALP [138]. It could be envisaged that the introduction of a sample containing phosphate would inhibit ALP. The reactions occurring using the bienzymatic approach are given as follows:

$$\text{Hydroquinone phosphate} \xrightarrow{\text{ALP}} \text{Hydroquinone} \quad (19)$$

$$\text{Hydroquinone} \rightarrow \text{Quinone} + 2e + 2H^+ \quad (20)$$

$$\text{Quinone} \xrightarrow{\text{GOD}} \text{Hydroquinone} \quad (21)$$

$$\text{Hydroquinone} \rightarrow \text{Quinone} + 2e + 2H^+ \quad (22)$$

The key strength of alkaline phosphatase is that it is relatively non-specific in terms of the nature of the phosphate ester upon which it can act. This provides a significant operational advantage over some of the other enzymes in that it can be directly coupled with a wider range of secondary enzymes. Similar amplification

as explained in the previous paragraph could be brought about by using phenyl phosphate as the substrate for ALP which can now be combined with the enzyme polyphenol oxidase, PPO [139]. The hydrolysed product phenol produced by ALP reacts with PPO to yield the orthoquinone which is reduced at a cathodic potential of −0.2 V and will be free from interference effects. The current for the reduction will again be inversely related to phosphate concentration as it acts as an inhibitor for the AP catalysed ester hydrolysis. The major advantage in this instance is that oxidation of other matrix constituents can often be avoided. The PPO component therefore serves to improve both selectivity and sensitivity providing a detection limit of 0.2 ppm (2×10^{-6} mol L^{-1}) for phosphate [126]. The reactions corresponding to this approach of detection is as follows:

$$\text{Phenyl phosphate} \xrightarrow{\text{ALP}} \text{Phenol} \quad (23)$$

$$\text{Phenol} \xrightarrow{\text{PPO}} o\text{-Quinone} \quad (24)$$

$$o\text{-Quinone} + 2e^{+} + 2H^{+} \rightarrow \text{Hydroquinone} \quad (25)$$

There are a few potentiometric biosystems for phosphate [140,141]. One of the approaches has successfully harnessed the ALP enzyme-induced cleavage of phosphate. In this case the ester used as the substrate for ALP was (*o*-carboxyphenyl phosphate), which gave rise to salicylate as the hydrolysis product. The reaction was potentiometrically sensed with the help of salicyclate membrane electrodes. The phosphate inhibited competitively the formation of salicylate to an extent inversely proportional to the concentration of added phosphate. The detection limit was 0.05×10^{-3} mol L^{-1}. The method was successfully applied to the determination of phosphate in blood serum [140].

Phosphate enzyme systems are found to be amenable for electrochemical interrogation and are currently widely exploited in biomedical research. It should also be possible to transfer the technology to environmental analysis as well. The multi-enzyme assemblies are however complex, expensive and less stable and will be subjected to fouling by different enzymatic products. Hence in order to make use of an enzymatic method of analysis advantageously, for onsite screening sites, it will be preferable to make use of screen printed electrodes that can be disposed of after each analysis. Table 2 provides the performance of some enzyme based sensors in terms of analytical parameters like detection limit, sensitivity, response time, storage life, etc.

8. Conclusion

Over the decades many analytical protocols have been developed for the sensing of inorganic phosphate ions. Each method has its own limitations as mentioned in this review. Potentiometric systems offer the simple requirement of instrumentation and low production costs and are suitable for field based analysis, environmental monitoring, clinical analysis and remote sensing. However, very small concentration changes lead to only less significant changes in the potential .Hence frequent recalibrations are mandatory. Temperature should be carefully controlled to avoid potential drifts arising from temperature fluctuations. The reference electrode used for the measurements should be highly reliable. These are the stumbling blocks while implementing the potentiometric sensors for implanting applications and remote sensing where the sensors need to be kept inside the measuring locations for prolonged periods. The influence of supramolecular chemistry is seen in the synthesis of a wide variety of signal compounds appended with phosphate binding groups. Synthetic organic chemists are successful in preparing heteroditopic ligands that can simultaneously detect an anion and a cation. Similarly a number of ferrocenyl dendrimers and silane based ferrocenyl dendrimers with structural complexities have been synthesised as anion receptors. Though a lot of efforts have been invested in this direction, it is generally observed that the analysis of anions can only be carried out in non aqueous solvents with these supramolecular compounds and in most of the cases these compounds exhibit recognition towards a number of anions. Further these reports mainly showcase the organic synthetic skills of the researchers identifying supramolecules for anion sensing. One of the widely used techniques that are often used for field based measurements is the electrochemical sensing of phosphate using ammonium molybdate. Potential dependent selectivity combined with the portability and miniaturisation capabilities make the electrochemical determination of phosphate using molybdophosphate highly versatile. Literature also contains reports about some non-conventional techniques that are amenable for phosphate sensing. These techniques need further rigorous evaluation and input in terms of their suitability for field measurements is still required. Enzymatic methods can become more popular analytical techniques for the determination of phosphate when the number of enzymes participating in the detection scheme is less. Further, it is preferable to use screen printed electrodes for onsite measurements and biomedical research when the stability and life of the biosensor becomes questionable. However, it is possible that researchers can develop genetically engineered enzymes that can exhibit biocatalytic activity for prolonged periods. Advanced research is needed in the direction of enzyme based sensing for applications in biomedical research for developing online monitoring systems, implantable sensors etc. In the case of environmental analysis, rugged and stable sensing systems are required. Presently, the analysis of phosphate, based on the electrochemical reduction of molybdophosphate and Co/Co oxide systems are considered to be successful for field measurements while other methods need a lot of rigorous validations. Selective sensing of phosphate is an ongoing challenge. A closer look at the problems jointly by sensor chemists and anion coordination chemists is required to design ionophores for the selective sensing of phosphate ions devoid of interferences from anions like nitrate and sulphate which are often difficult to decouple during phosphate sensing.

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