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Potentiometric Sensing of Aqueous Phosphate by Competition Assays Using Ion-Exchanger Doped-Polymeric Membrane Electrodes as Transducers

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Using Zn²⁺-BPMP or Cu²⁺-BPMP as a receptor and o-mercaptophenol as an indicator, potentiometric sensing of aqueous phosphate by competition assays was achieved. With attractive features of portability, low cost and resistance to interferences from turbidity and color, this sensor was successfully used for phosphate detection in biological and water samples.

Anions play important roles in numerous biological processes and environmental systems,¹ and plenty of efforts have been devoted to developing recognition and sensing platforms for anions.² Among various anions, the inorganic phosphate anions, which are ubiquitous in biological and environmental systems, attracted almost the most attention. As the composition of DNA, phosphate anions are one of the most important constituents of living systems.³ In addition, phosphate ions and their derivatives play important roles in diverse cellular functions such as signal transduction and energy storage.³⁻⁴ The abnormal levels of phosphate in body fluids, such as blood serum, urine and saliva, are the markers of irregular physiological functions.^{2b} Phosphate anions are not only very significant for biological systems, they may have deleterious effects in aquatic ecosystems. An excessive concentration of phosphate ions in aquatic system will promote eutrophication, which is well-known as the most widespread water-quality problem.⁵ To some extent, phosphate anions are convenient tracers of organic pollution in environmental waters.^{1b} An understanding of phosphate levels in biological fluids and environmental waters can provide useful information about several diseases, eutrophication, and many other problems.⁶ Thus, it is vital to detect phosphate in biological fluids and water. The standard method for phosphate detection in water is a colorimetric

technique based on the formation of a blue colored complex between phosphate and molybdate ions⁷. Besides that, many kinds of methods with various readout strategies have been developed for phosphate determination, such as colorimetry,^{3,8} fluorometry,^{1b,2b,6b,9} chemiluminescence,¹⁰ SPR¹¹ and electroanalysis.^{5,12} However, these methods usually suffered from the interference with turbidity and color, or dependence on large laboratory instruments. Further developing simple, sensitive and cost-effective approaches for phosphate detection in aqueous solutions is a worthwhile yet challenging task.⁵

Polymeric membrane ion-selective electrodes are a type of sensor based on the heterogeneous ion-transfer processes at plasticized polymeric membrane/water interfaces facilitated by ionophores within the membrane.¹³ The response behaviors of these electrodes are controlled by the electrochemical processes of ion-ionophore complexations at the very interfaces. With attractive features of portability, low cost, easy of miniaturization and integration, and resistance to interferences from turbidity and color usually encountered for real samples analysis, polymeric membrane ion-selective electrodes have been developed for more than 60 ions and found successful real-world applications in many important fields such as blood electrolyte analyses and noninvasive microtests.¹⁴ Although cations sensitive electrodes have been well developed and widely used in many fields, developing anions selective electrodes are more challenging because the high hydration energies prevents them from being extracted into the membrane phase efficiently. At the forefront of the Hofmeister series, phosphate anions are one of the most challenging targets for anion recognition chemistry. Using amide, urea and thiourea as promising hydrogen bonding donors, a number of receptors for phosphate recognition in organic phase have been developed.¹⁵ However, they are not necessarily good ionophores for phosphate because the complexations at the oil/water interfaces may be deteriorated owing to the weakened hydrogen-bonding interactions in the presence of highly polar water.¹⁶ Moreover, most of these receptors are not good components of the membrane owing to their poor solubility in the plasticized poly(vinyl chloride). To

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Scheme 1 Response mechanism of the proposed competition assays. All the pK_a and $\text{Log } P$ values were calculated by ACD/Lab 12.

achieve potentiometric phosphate sensing, new mechanism should be developed.

In the work present here, we report a potentiometric strategy for aqueous phosphate detection by competition assays at neutral pH values (Scheme 1). After incubation the receptor-indicator ensembles with phosphate, the indicators will be displaced from the receptor, then potential signals can be obtained by initiating the oxidation reaction of the indicators using HRP and H_2O_2 . When phosphate is absence in the samples, no signals will be obtained because the indicators are captured by the receptors. In assembling the sensor, receptors and indicators are necessary. For constructing the receptors, we took advantage of metal-ligand and electrostatic interactions, which are highly favourable and can occur even in polar media. Moreover, different from small spherical ions with strong coordination abilities,¹⁷ for polyatomic phosphate ions, receptors with groups spatially complementary with the targets are desired.¹⁸ The metal complexes of 2,6-bis(bis(2-pyridylmethyl)aminomethyl)-4-methylphenol (H-BPMP) were used as receptors considering that phosphate anions and catechols could bind to the dinuclear complexes by bridging the two metal ions.¹⁹ And this is exactly why they have been used as the mimics of the active sites of phosphatase and catechol oxidases.¹⁹ Species have similar structures and coordination sites with catechols were chosen as indicators. These catechols, *o*-mercaptophenol, *o*-benzenedithiol and their derivatives are promising potentiometric reporters according to our previous work.²⁰ Using ion-exchanger doped polymeric membrane electrodes as transducers, potentiometric detection of aqueous phosphate can be achieved. The sensor is easy to assemble and shows a high sensitivity and excellent selectivity for phosphate ions over other anions. The utility of the sensor was demonstrated by detection of phosphate in urine, saliva and mineral waters.

For the synthesis of H-BPMP, bis(2-pyridylmethyl)amine and

Scheme 2 Synthesis path of the H-BPMP. Reagents and conditions: (i) THF; (ii) Triethylamine.

2,6-bis(chloromethyl)-4-methylphenol were first synthesized according to the literature (Scheme S1 and S2, see Fig. S1 and S2 for 1H -NMR and ^{13}C -NMR, ESI+).²¹ H-BPMP was obtained by alkylation of the amine as shown in Scheme 2 (see Fig. S3 for 1H -NMR and ^{13}C -NMR, ESI+).¹⁹ With the ligand in hand, proof-of-concept experiment was performed to verify the proposed mechanism. As shown in Scheme 1, the best situation for discrimination is only the phosphate can displace indicators from receptors, with subsequent revival of potential signal. However, even in this situation, other anions may also interference with the detection. These anions could induce potential response directly on the ion-exchanger doped polymeric membrane electrodes with a selectivity reflects the Hofmeister series. To exclude the interference from these unfavourable anions, the signals from the oxidation of the displaced indicators were used for phosphate detection. After mixing H-BPMP (50 μM), $Zn(ClO_4)_2$ (100 μM), and *o*-mercaptophenol (indicator 4, 10 μM) in 20 mM HEPES buffer ($\text{pH}=7.0$), phosphate (50 μM) or chloride (250 μM) was added. Then H_2O_2 (10 mM) and horseradish peroxidase (HRP, 0.1 U) were used to initiate the oxidation reaction and the potential

Fig. 1 Potential responses of the TDMACl-doped polymeric membrane electrodes to *o*-mercaptophenol displaced from the metal-BPMP complexes by 50 μM phosphate or 250 μM chloride in the presence of 10 mM H_2O_2 and 0.1 U HRP. The sample medium is 20 mM HEPES buffer ($\text{pH}=7.0$), the same below.

Fig. 2 Potential responses of the TDMACI-doped polymeric membrane electrodes to 50 μM indicators (I) in the presence of 10 mM H_2O_2 and 0.1 U HRP.

responses were recorded (the concentration of H_2O_2 and HRP was optimized to obtain high sensitivity, see Fig. S4, ESI[†]). Fig. 1 shows that signals can be obtained only from the system containing phosphate, thus illustrating the feasibility of the proposed mechanism.

After confirming the mechanism, we have to find indicators to be coupled to the receptors to optimize the selectivity and sensitivity of the proposed sensor. Indicators were chosen on the basis of their potential signals in the process of oxidation and their binding stabilities with the receptors. From these perspectives, 6 kinds of species containing two phenolic hydroxy/sulfhydryl groups with appropriate distances capable of bridging the two metal centers of the receptors were investigated. Although the two coordination groups in the indicators cannot deprotonate completely in pure HEPES buffer ($\text{pH}=7.0$), the metal-BPMP complexes may promote the dissociation process as reported previously.³ As shown in Scheme 1, indicators 1-3 are catechols containing two adjacent hydroxyls, indicator 4 is *o*-mercaptophenol in which a sulfhydryl neighbours a hydroxyl, and indicators 5 and 6 are benzenedithiols containing two adjacent sulfhydryl groups. Indicators containing more sulfhydryl groups will show higher binding affinities with the receptors, considering the strong affinity of a soft thiolate to transition metal ions. Fig. 2 shows that the potential responses of the polymeric membrane electrodes to the indicators reflect their lipophilites and acidities, and this is in consistent with previous work.^{14b,20} When H_2O_2 and HRP are added into the system, catechols (indicators 1-3) will produce C-C and C-O coupling products with large lipophilites, and thus the potentials are more negative.^{20a} For indicators 4-6, S-S coupling products with less reporter groups (sulfhydryl groups) will be favourable, this is why the potential responses are reversed.^{20b} The potential responses to the oxidation reactions indicate that indicators with electron-donating groups are good substrates for HRP (see indicators 3 and 6). When indicators 1-3 were used for phosphate detection, poor sensitivity was obtained. Although indicators 5 and 6 are promising in sensitivity, they bind the

Fig. 3 Potential responses of the TDMACI-doped polymeric membrane electrodes to 10 μM indicator 4 in the presence of (a) Zn^{2+} -BPMP and (b) Cu^{2+} -BPMP at different concentrations, and to (c) indicator 4 at different concentrations; (d) Calibration curve for (c). Each error bar represents one standard deviation of 3 replications, the same below.

receptors so strong that it is difficult to displace them from the receptors by the target. Indicator 4 is used in the following experiments for sensitive and selective phosphate detection. The components of the membrane were optimized (Fig. S5, ESI[†]), the NPOE plasticized membrane containing TDMACI as the recognition element shows the best sensitivity, this is in accordance with our previous work.²⁰

Two transition metal ions were used here for constructing the receptors, Zn^{2+} has been reported to have strong binding stability with phosphate and Cu^{2+} displays strong binding tendencies towards anions because their electronic configuration ensures high ligand field stabilization effects.^{8,9} The metal ions in the receptors present some geometrical preferences, thus imparting selective binding tendencies towards anions of given shapes, such as phosphate and the indicators.¹⁸ For efficient displacement, the association constants (K_a) for the receptors and phosphate should be larger than that for the receptors and indicators. To confirm it, the K_a for receptors and indicator 4 were determined using titration experiments (Fig. 3). And the results for phosphate and receptors were estimated from the competition assays of phosphate, where a solution of the receptor/indicator couple was titrated with different concentrations of phosphate (Fig. 4).

Tab. 1 The association constants (K_a) and calculated Gibbs energies (ΔG) for the binding of indicator 4 or phosphate ions to Zn^{2+} -BPMP and Cu^{2+} -BPMP in 20 mM HEPES buffer ($\text{pH}=7.0$).

Host/ Guest	Zn^{2+} -BPMP		Cu^{2+} -BPMP	
	K_a 10^4 M^{-1}	ΔG kJ mol^{-1}	K_a 10^4 M^{-1}	ΔG kJ mol^{-1}
Indicator 4	8.4 ± 0.5	-27.66	10.7 ± 0.4	-28.24
HPO_4^{2-}	10.4 ± 0.6	-28.16	11.3 ± 0.8	-28.37

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Fig. 4 Potential responses of the TDMACI-doped polymeric membrane electrodes to the oxidation of indicator 4 displaced from (a) Zn^{2+} -BPMP and (b) Cu^{2+} -BPMP by phosphate at different concentrations; (c) Calibration curves; (d) Signals for the detection of 50 μM phosphate and 250 μM other anions.

It can be seen from Tab. 1 that phosphate anions bind the receptors more tightly than the indicator, which build the foundation for competition assay.

For phosphate detection, 50 μM Zn^{2+} -BPMP or Cu^{2+} -BPMP was mixed with 10 μM indicator 4 first, then phosphate at different concentrations was added, the potential responses of the polymeric membrane electrodes were recorded after initiation of the oxidation reaction by H_2O_2 and HRP. Phosphate can be detected in the range of 3 ~ 50 μM (detection limit: 1 μM , Fig. 4(a) and (c)) and 3 ~ 50 μM (detection limit: 0.5 μM , Fig. 4(b) and (c)) when Zn^{2+} -BPMP or Cu^{2+} -BPMP was used as the receptor, respectively. It can be observed that the sensor constructed using Cu^{2+} -BPMP are more sensitive and selective, this is in consistent with the binding affinity. The larger the K_a is, the lower the amount of receptor needed to bind the indicator, and the lower the concentration of phosphate required to displace the reporter from the receptor. Sensors constructed using other metal ions (Mg^{2+} and Ni^{2+}) show poor sensitivity, owing to their inability to bind the indicator with high affinity (see Fig. S6, ESI[†]). The Zn^{2+} -BPMP based sensor can be used for at least five times without significant deterioration in sensitivity (The polymeric membrane electrodes were soaked in 0.1 M NaCl solution for 5 min between each detection). However, considering that the polymeric membrane is very cheap, single usage is recommended (see Fig. S7, ESI[†]). The proposed sensor exhibits excellent selectivity towards phosphate ions over other anions, the tolerant concentration of SO_4^{2-} , Cl^- , Br^- , NO_3^- , ClO_4^- , HCO_3^- and acetate was at least 250 μM . It should be noticed that the interference from SO_4^{2-} is a little bigger than that from other anions. These results reflect the strong binding stability between SO_4^{2-} and the receptors, originating from its high charges and similarly tetrahedral shapes with phosphate. To demonstrate the practical utility of the sensor, we applied it to

Tab. 2 Analytical results (mean $\pm$ standard deviation, $n = 3$) for the detection of phosphate in biological and water samples.

Samples	Concentration of phosphate in samples	
	This work	The standard method
Mineral water 1	$8.45 \pm 0.51 \mu\text{M}$	$8.80 \pm 0.07 \mu\text{M}$
Mineral water 2	$7.53 \pm 0.35 \mu\text{M}$	$7.23 \pm 0.09 \mu\text{M}$
Mineral water 3	$6.68 \pm 0.32 \mu\text{M}$	$6.58 \pm 0.05 \mu\text{M}$
Human urine 1	$42.50 \pm 2.15 \text{ mM}$	$41.80 \pm 0.02 \text{ mM}$
Human urine 2	$40.00 \pm 1.95 \text{ mM}$	$40.50 \pm 0.02 \text{ mM}$
Human urine 3	$36.58 \pm 0.95 \text{ mM}$	$37.00 \pm 0.03 \text{ mM}$
Human saliva 1	$5.20 \pm 0.09 \text{ mM}$	$5.30 \pm 0.01 \text{ mM}$
Human saliva 2	$4.20 \pm 0.15 \text{ mM}$	$4.10 \pm 0.02 \text{ mM}$
Human saliva 3	$4.15 \pm 0.25 \text{ mM}$	$4.35 \pm 0.04 \text{ mM}$

the detection of phosphate in mineral water, human urine and saliva samples. The concentrations of phosphate in water, human urine, and serum samples obtained by our method are in good agreement with those measured by the standard method (Tab. 2). These results show that the proposed sensor is promising for phosphate detection in biological and water samples with good sensitivity and selectivity.

In summary, a potentiometric platform for aqueous phosphate detection by competition assays was developed. Using Zn^{2+} -BPMP or Cu^{2+} -BPMP as receptor and *o*-mercaptophenol as indicator, sensitive and selective phosphate detection was achieved. With attractive features of portability, low cost and resistance to interferences from turbidity and color, this sensor was successfully used for phosphate detection in biological and water samples.

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