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A novel fluorescence method for detection of phosphate anions based on porphyrin metalation

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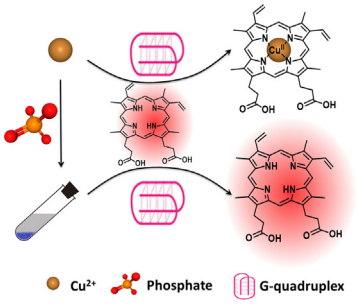
HIGHLIGHTS

- A phosphate biosensor is developed based on porphyrin metalation.
- G4 had the greatest effect on increasing the fluorescence intensity of NMM.
- All materials used in the process were commercially available.
- The sensitivity of biosensor is very high with the limit of detection 44 nM.

GRAPHICAL ABSTRACT



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ABSTRACT

In this work, a novel fluorescence method for the detection of phosphate anions (PO_4^{3-}) was developed based on porphyrin metalation. Through catalysis by G-quadruplex (G4), Cu^{2+} could insert into the porphyrins to quench their fluorescence. G4 simultaneously improved the fluorescence of the porphyrins but not that of Cu^{2+}-porphyrin. In the absence of PO_4^{3-}, the porphyrins were metallized by Cu^{2+}, and no fluorescence was observed. In the presence of PO_4^{3-}, PO_4^{3-} could coordinate with Cu^{2+} to prevent porphyrin metalation. Free porphyrin could bind with G4 to emit strong fluorescence. By comparing four common porphyrins, we found that G4 had the greatest effect on increasing the fluorescence intensity of *N*-methylmesoporphyrin IX (NMM). Thus, NMM/G4 was chosen for the design of a biosensor. Under optimal experimental conditions, this method showed high sensitivity and satisfactory selectivity for PO_4^{3-} with a detection limit of 44 nM in a linear range of 0.01–1.0 μM. The recovery experiments showed recovery rates of 93.75–106.00%, suggesting a great potential for measuring PO_4^{3-} in real samples.

1. Introduction

Phosphorus is a fundamental element that is necessary for the growth and development of living organisms. It is the building block of nucleic acids, ATP and cell membranes [1,2] and plays an important role in signaling pathways through phosphorylation and dephosphorization [3]. The stable form of phosphorus is PO_4^{3-}, which is considered to be the main carrier of phosphorus. *In vivo*, abnormal levels of PO_4^{3-} in body fluids (such as serum, saliva or urine) may result in physiological dysfunction [4]. For example, PO_4^{3-} deficiency can result in muscle weakness, impaired leukocyte function and irregular bone mineralization [5,6]. However, a large

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increase in PO_4^{3-} levels can cause abnormal kidney function [7,8]. *In vitro*, PO_4^{3-} is the main component of plant fertilizer. Excessive use and discharge of PO_4^{3-} can cause serious environmental problems, such as water eutrophication [9,10]. Water eutrophication is primarily responsible for red tide in oceans and algae blooms in freshwater bodies, which can bring great economic loss, environmental pollution and social disruption. For example, a cyanobacterial bloom event occurred in Taihu Lake in 2007 [11,12]. Therefore, it is very important to monitor changes in PO_4^{3-} concentration.

To date, many approaches have been developed for PO_4^{3-} analysis, including instrumental methods (such as gas chromatography, high-performance liquid chromatography and Raman spectroscopy) [13-15], organic small molecule probes [16] metal-organic frameworks (MOFs) [17], electroanalytical methods [18] and enzymatic detection [19]. These methods have presented good performances in the detection of PO_4^{3-}. However, they usually require expensive instruments and tedious synthesis processes. Porphyrins are compounds with unique fluorescence spectra [20]. When the metal ions insert into the porphyrin ring (called porphyrin metalation), the fluorescence spectra of the porphyrins changes greatly. This reaction has been used for the development of biosensors with simple operation, good repeatability and high accuracy [20,21]. For example, the Wang group utilized the insertion of Cu^{2+} into protoporphyrin IX (PPIX) to cause a fluorescence decrease and thus detect Cu^{2+} [22]. Yuxi Xu *et al.* developed a method of detecting Cd^{2+} based on the insertion of Cd^{2+} into 5,10,15,20-tetrakis(1-methyl-4-pyridinio) porphyrin tetra (*p*-toluenesulfonate) (TMPyP) [23]. Recently, we developed a new method for the detection of Pb^{2+} based on the porphyrin metalation of Pb^{2+} [24]. However, porphyrin metalation is rarely reported for the determination of PO_4^{3-}.

Recently, DNA biosensors have attracted much attention because DNA is easy to synthesize and stable [25,26]. Among them, G-quadruplex structure (G4) with special properties is often employed to design DNA biosensors. The G4 is a nonclassical DNA structure that is assembled from G-quartets through π-π stacking. The G-quartet is a quasi-planar structure formed by four guanines with Hoogsteen-type base pairing [27]. In aqueous solution, porphyrins are in the form of micelles that have low fluorescence due to their poor water solubility. The porphyrins can insert into G4 through π-π stacking between the porphyrin ring and G-quartets, which can greatly increase their fluorescence [28]. Due to this phenomenon, porphyrin/G4 is widely used as a reporter in the development of biosensors [29]. Meanwhile, G4 has another function as DNAzyme to catalyze porphyrin metalation [20]. Liu group found that T30695 (G4 DNA) can accelerate Cu^{2+} and Zn^{2+} insertion into mesoporphyrin IX (MPIX) [21]. We recently discovered that G4 can also catalyze the insertion of Pb^{2+} into protoporphyrin IX (PPIX) [24]. Here, we developed a new method for detecting PO_4^{3-} by combining porphyrin metalation with the porphyrin/G4 pair.

2. Experimental section

2.1. Reagents and materials

Porphyrin compounds, *N*-methylmesoporphyrin IX (NMM), protoporphyrin IX (PPIX), mesoporphyrin IX (MPIX), 5,10,15,20-tetrakis (1-methyl-4-pyridinio) porphyrin tetra (*p*-toluenesulfonate) (TMPyP), were purchased from Sigma-Aldrich (St. Louis, MO, USA). G-quadruplex DNA PS5.M (5′-GTGGGTCATT GTGGGTGGGTGTGG-3′) was reported to catalyze the insertion of Cu^{2+} into porphyrins by the Sen Group in 1997 [30]. This DNA was ordered from Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). $Cu(NO_3)_2$, Na_3PO_4, K_2CO_3, KCl, Na_2SO_4, Na_2SO_3, KBr, KNO_3, and 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China). All chemicals were of at least analytical grade. All aqueous solutions were prepared with deionized water (Milli-Q, resistance 18.2 MΩ/cm) in this experiment.

2.2. Instrumentation

The optimization of Cu^{2+} concentration and reaction time of Cu^{2+}-porphyrin formation were carried out on an F-7000 spectrometer (Hitachi, Japan). The instrument setting of F-7000 was as follows. The excitation wavelength was 399 nm, and the emission wavelengths ranged from 550 nm to 650 nm. The slit widths of both the excitation wavelength and emission wavelength were 10 nm. The photomultiplier tube (PMT) detector voltage was 500 V. The SpectraMax iD3 Multi-Mode Microplate Reader (USA) was used for other fluorescence measurements. The instrument setting of SpectraMax iD3 was as follows. The excitation wavelength was 399 nm, and the emission wavelength was from 550 nm to 710 nm. The photomultiplier tube (PMT) detector voltage was a medium voltage.

2.3. Fluorescence measurement

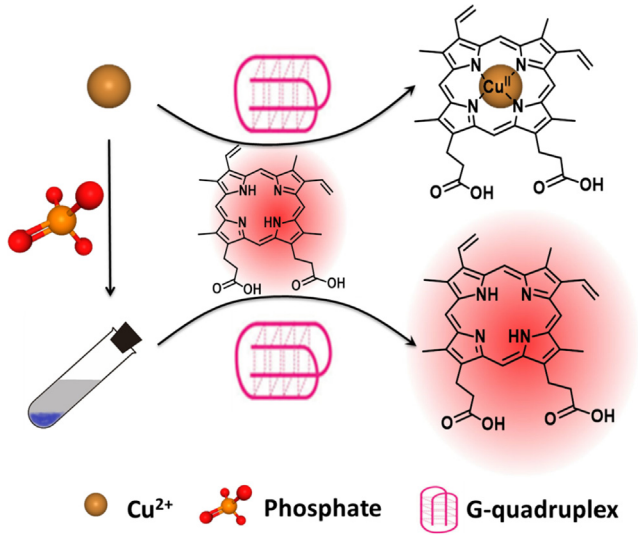
Forty mM HEPES buffer (pH 7.0) containing 25 mM KCl and 100 mM NaCl was prepared for all experiments. The Cu^{2+} concentration optimization experiment was performed as follows. First, 250 nM DNA and 3 μM NMM were added to HEPES buffer. Then, different concentrations of Cu^{2+} (from 1.0 μM to 8.0 μM) were added. The mixture was incubated at 25 °C for 1 h to ensure the complete porphyrin metalation. Finally, the fluorescence was measured on an F-7000 spectrometer.

The PO_4^{3-} detection experiment was carried out as follows. First, different concentrations of PO_4^{3-} (from 0 μM to 3.0 μM) were mixed with 4 μM Cu^{2+} in HEPES buffer. Then, the sample was incubated at 25 °C for 1 h to ensure that PO_4^{3-} and Cu^{2+} underwent a complete reaction. Second, 250 nM DNA and 3 μM NMM were added. Then, the mixture was incubated at 25 °C for 40 min to complete porphyrin metalation. Finally, the fluorescence spectra were recorded on a SpectraMax iD3 Multi-Mode Microplate Reader. We also tried to mix all the components together rather than mix the PO_4^{3-} and Cu^{2+} first. However, the results were not ideal. The reason might be that the coordination ability of Cu^{2+}-porphyrin was very strong, and this coordination affected the bonding of the PO_4^{3-} to Cu^{2+}.

3. Results and discussion

3.1. The detection principle of the method

As early as 1996, Yingfu Li and Dipankar Sen found that G4 could catalyze the insertion of Cu^{2+} into porphyrin [31]. Once Cu^{2+}-porphyrin forms, the fluorescence of porphyrin is quenched. Besides, PO_4^{3-} can strongly coordinate with Cu^{2+} to form a Cu^{2+}-PO_4^{3-} complex [32]. Therefore, PO_4^{3-} can effectively inhibit the formation of Cu^{2+}-PO_4^{3-} to retain the fluorescence of porphyrin by binding with Cu^{2+}. The fluorescence of free porphyrin can be further improved by G4. Based on these phenomena, we developed a method for detecting PO_4^{3-}. The detection principle is shown in Scheme 1. In the absence of PO_4^{3-}, porphyrin metalation could occur to form Cu^{2+}-porphyrin under the catalysis by G4. Because Cu^{2+}-porphyrin is not fluorescent, the fluorescence was not enhanced even in the presence of G4. Thus, no fluorescence was observed. When PO_4^{3-} was added, it could bind with Cu^{2+} to reduce the number of free Cu^{2+} ions in the solution. Therefore, some porphyrin was



Scheme 1. Schematic illustration of a label-free fluorescence detection method for PO_4^{3-} based on porphyrin metalation and coordination between PO_4^{3-} and Cu^{2+}.

not allowed to form Cu^{2+}-porphyrin. Free porphyrin could bind with G4 to emit strong fluorescence. With the increase in PO_4^{3-}, the fluorescence intensity might increase accordingly, so PO_4^{3-} could be detected.

3.2. The optimization of porphyrins

Porphyrins are a key factor in the metalation of Cu^{2+}-porphyrin because they may have different reactivities to Cu^{2+}. We selected four common porphyrin compounds in this experiment. They were *N*-methylmesoporphyrin IX (NMM), protoporphyrin IX (PPIX), mesoporphyrin IX (MPIX), and 5,10,15,20-tetrakis (1-methyl-4-pyridinio) porphyrin tetra (*p*-toluenesulfonate) (TMPyP). NMM, PPIX and MPIX are anionic porphyrins with two carboxyl groups, while TMPyP is a cationic porphyrin (**Fig. 1**). NMM itself is a methylated porphyrin; one hydrogen atom on the porphyrin ring of NMM is replaced by a methyl group.

Then, we carried out four treatments for each porphyrin: porphyrin, porphyrin + G4, porphyrin + G4 + Cu^{2+}, and porphyrin + G4 + Cu^{2+} + PO_4^{3-}. By comparing porphyrin and porphyrin + G4 (**the black and red curves in Fig. 2a**), we found that G4 had the greatest effect on the fluorescence of NMM, which increased by approximately 3.4 times (from 0.8×10^6 to 2.7×10^6). The porphyrin with the second largest fluorescence increase was MPIX, which exhibited a near 2.0-fold increase (from 3.4×10^5 to 6.6×10^5) (**the black and red curves in Fig. 2c**). However, the fluorescence enhancement of PPIX and TMPyP was very weak. This result suggested that NMM/G4 might be a better reporter in this biosensor design. When Cu^{2+} was added, we found that the fluorescence of all four porphyrins was quenched by Cu^{2+}, which implied that all four porphyrins could undergo porphyrin metalation with Cu^{2+} (**the red and blue curves in Fig. 2**). According to the experimental principle, when PO_4^{3-} was added, the fluorescence of all porphyrins should have been recovered, but this was not the case. In fact, there was a large fluorescence recovery for NMM, a slight increase for PPIX, and almost no change in the fluorescence of MPIX and TMPyP (**the blue and pink curves in Fig. 2**). The reason might be that one hydrogen atom on the porphyrin ring of NMM was replaced by a methyl group to result in a higher molecular energy that was analogous to a transition-state [31,33]. Thus, Cu^{2+}-NMM might be more unstable than other metallic porphyrins. Therefore, PO_4^{3-} could recover the fluorescence of NMM more easily. Therefore, NMM was selected for the following experiments.

3.3. Optimization of the Cu^{2+} concentration

According to the experimental principle, the concentration of Cu^{2+} was another key factor in PO_4^{3-} detection. Therefore, the Cu^{2+} concentration was optimized by observing the fluorescence changes at different Cu^{2+} concentrations. The results are shown in **Fig. 3.** In the beginning, the fluorescence signal decreased gradually as the concentration of Cu^{2+} increased. When the Cu^{2+} concentration increased to 4 μM, the fluorescence changes slowed, and the fluorescence signal reached a plateau. Then, higher Cu^{2+} concentrations (up to 8 μM) were tested, and still, the fluorescence signal hardly changed. Because excessive Cu^{2+} would affect the sensitivity of the method, 4 μM Cu^{2+} was chosen for the following experiments.

3.4. Sensitivity of the proposed method

Next, the sensitivity, which is a critical index for PO_4^{3-} detection, was studied by PO_4^{3-} titration experiments. The results showed that the fluorescence intensity increased gradually in the range of 0–3.0 μM PO_4^{3-} (**Fig. 4a**). Even at 0.01 μM PO_4^{3-}, there was a significant increase (**the red curves in Fig. 4a**). Subsequently, the fluorescence intensity of the maximum emission wavelength (615 nm) was researched for quantitative analysis. As shown in **Fig. 4b**, the fluorescence intensity increased rapidly when the PO_4^{3-} concentration ranged from 0.01 to 1.0 μM. Then, the fluorescence changes slowed and reached a plateau. We observed a good linear relationship between the fluorescence and PO_4^{3-} concentration at 0.01–1.0 μM (**inset of Fig. 4b**). The linear equation was $Y = 83717 + 1.167 \times 10^6 X$ ($R^2 = 0.996$), where Y and X represent the fluorescence and PO_4^{3-} concentration, respectively. This result suggested that the proposed method could detect PO_4^{3-} quantitatively. The limit of detection was estimated to be 0.044 μM (44 nM) based on the formula 3σ/slope. σ was the standard deviation, which was calculated by measuring the fluorescence of the blank solution at 615 nm. The

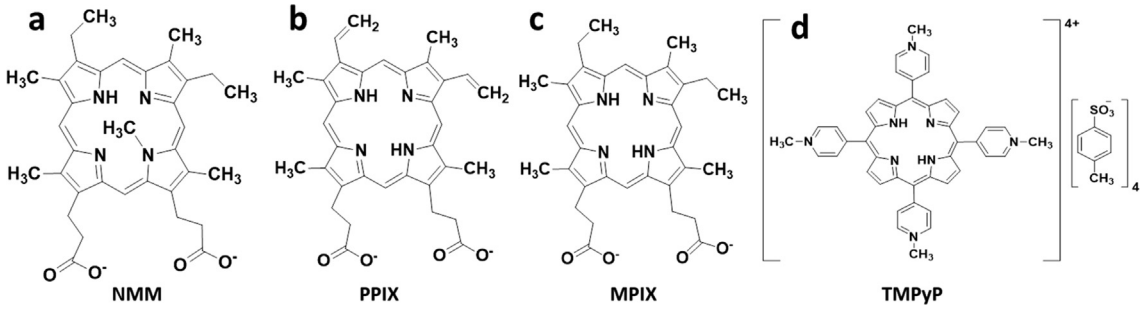


Fig. 1. The common porphyrin structures used in this experiment.

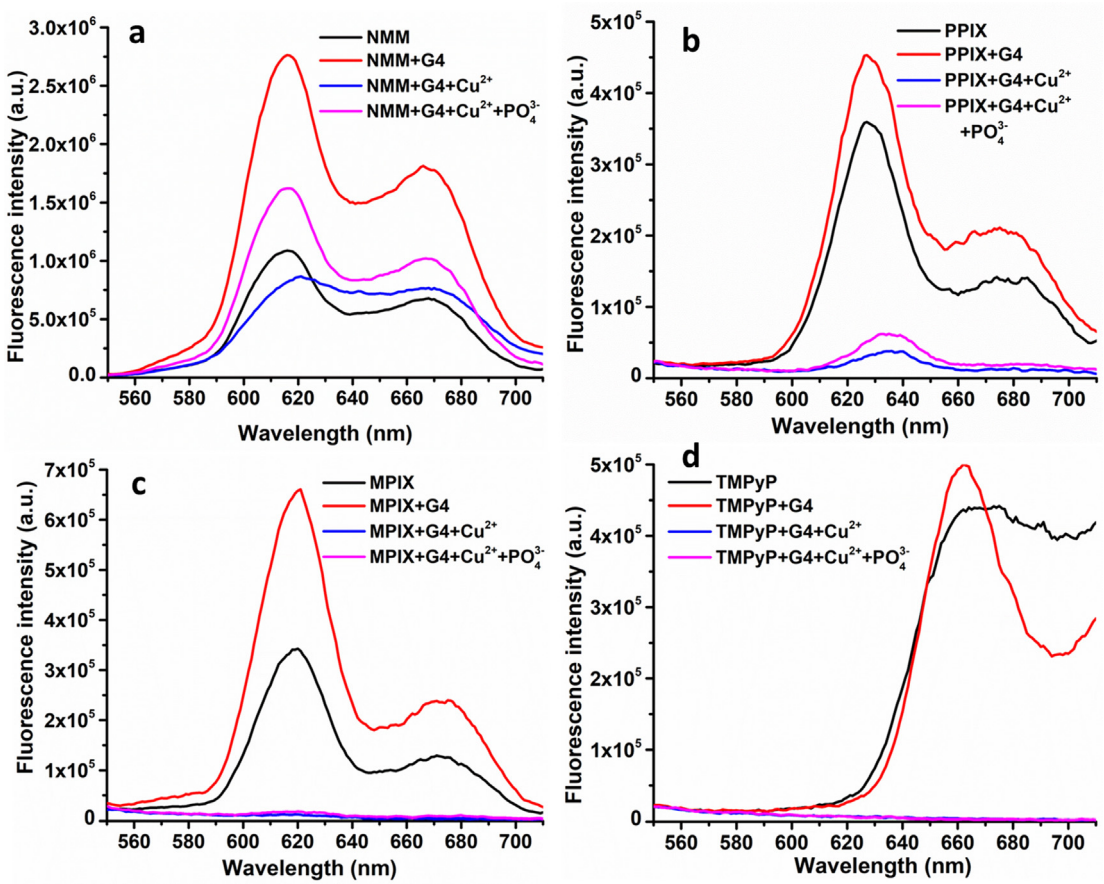


Fig. 2. Fluorescence emission spectra of four porphyrins: (a) NMM, (b) PPIX, (c) MPIX and (d) TMPyP under four different treatment conditions: free porphyrin (black curve), porphyrin + G4 (red curve), porphyrin + G4 + Cu^{2+} (blue curve), and porphyrin + G4 + Cu^{2+} + PO_4^{3-} (pink curve). The concentrations of porphyrins, DNA, Cu^{2+} and PO_4^{3-} were 3 μM, 250 nM, 3 μM and 0.6 μM, respectively.

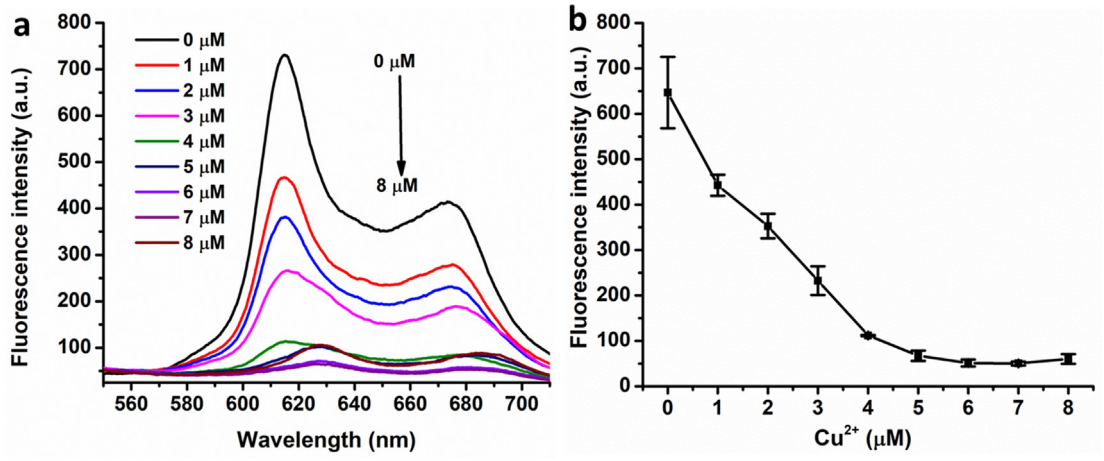


Fig. 3. (a) Fluorescence emission spectra of different Cu^{2+} concentrations (0–8 μM). (b) The fluorescence intensity change at the maximum emission wavelength (615 nm) under different Cu^{2+} concentrations.

slope was obtained from the linear equation. The Chinese government has issued a policy (GB8978-1996) to define the maximum allowable discharge concentration of PO_4^{3-} in industrial wastewater as 0.5 mg/L (approximately 5.2 μM). The detection limit of our method was far below this maximum allowable discharge standard. In addition, we compared the detection limit of our method with that of the other reported methods. The results showed that our method performed very well among them (Table 1). In addition, the "turn-on" response mode was another advantage of our method.

3.5. Selectivity of the proposed method

The selectivity was another important index for PO_4^{3-} detection, which was researched by comparing the responses of different anions. The results are shown in Fig. 5. Only PO_4^{3-} exhibited a strong

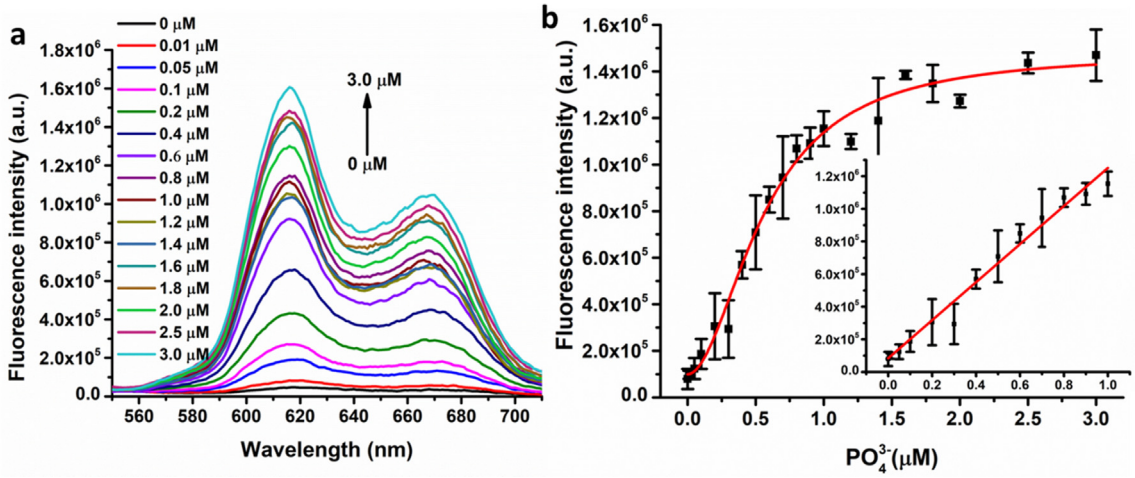


Fig. 4. (a) Fluorescence emission spectra of different PO_4^{3-} concentrations (0–3.0 μM). (b) The fluorescence intensity change at the maximum emission wavelength (615 nm) under different PO_4^{3-} concentrations. Inset: the signal change exhibited a linear relationship with PO_4^{3-} concentrations from 0.01 μM to 1.0 μM.

Table 1
Comparison of the sensitivity of our method and other methods.

Detection methods	Respond mode	Linear range (μM)	Limit of detection (nM)	Reference
Organic small molecule probe	Turn-on	10–150	65.6	[16]
Organic small molecule probes	Turn-on	Not reported	1721	[34]
Metal-organic frameworks probe	Turn-off	0.1–15	52	[35]
Metal-organic frameworks probe	Turn-on	5–150	1250	[17]
Enzyme probe	Turn-off	40–350	200	[36]
Enzyme probe	Turn-on	1–50	1000	[37]
Our method	Turn-on	0–1.0	44	This study

fluorescence recovery. Its fluorescence intensity was much greater than that of other anions. Other common anions had little response, and the fluorescence intensity was similar to that of the blank control (added H_2O). These results suggested that our method had good selectivity for PO_4^{3-}. The satisfactory selectivity was attributed to the strong coordination ability of Cu^{2+} and PO_4^{3-} [32]. Our results were consistent with those of other PO_4^{3-} probes that also use the binding between Cu^{2+} and PO_4^{3-} to recognize Cu^{2+} [32].

3.6. Determination of PO_4^{3-} in pond water

To investigate the feasibility of the proposed method in real samples, we carried out recovery experiments of PO_4^{3-} in pond water. The pond water came from the campus of Yangtze University and was simply filtered by gauze to remove large particulate impurities. First, different concentrations of PO_4^{3-} water samples (0.2 μM, 0.5 μM and 0.8 μM) were prepared through the standard addition method. Then, the PO_4^{3-} concentrations were quantitatively calculated from the linear regression equation mentioned above. The recovery rates were also calculated. The results are shown in Table 2, and the recovery rates were between 93.75 and 106.00%. The results suggested that our proposed method was reliable for measuring PO_4^{3-} in real pond water samples. It also suggested that the pond water of Yangtze University was not eutrophic and that the background concentration of PO_4^{3-} was very low.

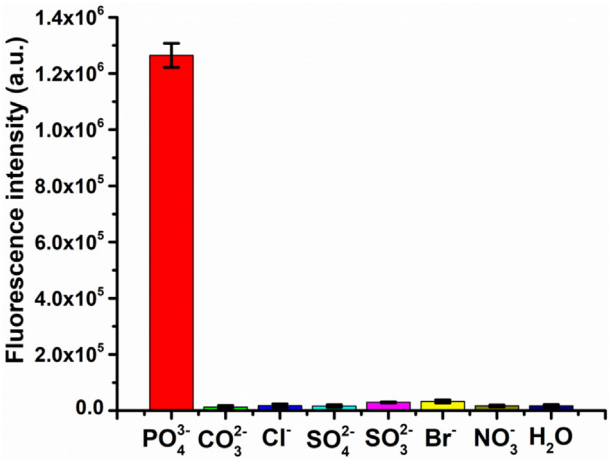


Fig. 5. The fluorescence intensity of different anions at the same concentration (2 μM).

Table 2
Determination of PO_4^{3-} in pond water.

Samples	PO_4^{3-} Added (μM)	PO_4^{3-} Found (μM)	Recovery (%)
1	0.00	undetectable	–
2	0.20	0.19 ± 0.01	95.00
3	0.50	0.53 ± 0.06	106.00
4	0.80	0.75 ± 0.05	93.75

4. Conclusions

In summary, a novel fluorescence method for the detection of PO_4^{3-} was developed based on porphyrin metalation. To our knowledge, no PO_4^{3-} probe based on porphyrin metalation has been previously reported. In this method, G4 was used not only to improve the fluorescence of porphyrins, but also as a DNAzyme to catalyze porphyrin metalation. All materials used in the process, including porphyrin and G4-DNA, were commercially available. Therefore, this method can be conveniently applied. By comparing

four common porphyrins, G4 showed the greatest effect on increasing the fluorescence intensity of NMM, suggesting NMM/G4 as a reporter in this biosensor. This method showed high sensitivity with a detection limit of 44 nM and satisfactory selectivity. Additionally, recovery experiments showed that this method had a great potential to detect PO_4^{3-} in actual samples.

CRediT authorship contribution statement

Xingping Zhang: Investigation, Writing – original draft, Methodology, Formal analysis, Validation. **Jiujun Wang:** Methodology, Formal analysis, Validation. **Yewen Juan:** Methodology, Formal analysis, Validation. **Hualin Yang:** Conceptualization, Project administration, Funding acquisition, Writing – review & editing. **Wei Wei:** Investigation, Funding acquisition, Writing – review & editing. **Jing Zhao:** Investigation, Conceptualization, Project administration, Funding acquisition, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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