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ARTICLE TYPE

# Phenanthroimidazole-based dizinc(II) complex as a fluorescent probe for pyrophosphate ion as generated in polymerase chain reactions and pyrosequencing†

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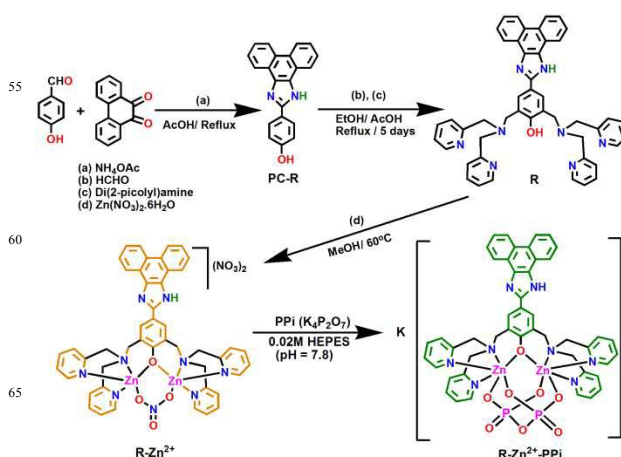
A highly selective and sensitive phenanthroimidazole tagged Mannich base type dizinc(II) fluorescent probe ( $R-Zn^{2+}$ ) has been developed for pyrophosphate ion (PPi) with a very low limit of detection (LOD) of 0.25 ppm; this also assesses PPi from DNA polymerization chain reaction (PCR).

Development of novel fluorescent probes for the detection of bio-relevant anions is a promising research field in sensor chemistry with significance in ecological and biomedical applications.<sup>1,2</sup> In particular, the detection of pyrophosphate ion (PPi) is essential because it is involved in several biological processes including cellular signal transduction, gene transcription and protein synthesis.<sup>3</sup> In light of this, detection of PPi is being investigated as a real time PCR method and vital tool in cancer research.<sup>4</sup> The selective detection of this biologically potent PPi is an important field.<sup>5</sup> Different methods based on hydrogen bonding, anions- $\pi$  interaction and metal-anion interactions (chelation) have been applied to the development of fluorescent probes for PPi,<sup>5,6</sup> phosphate ions (Pi),<sup>7</sup> adenosine triphosphate (ATP)/guanosine triphosphate (GTP),<sup>1c,8</sup> or phosphorylated peptide.<sup>9</sup> However, only a few of such probes are simple, accessible and water soluble.

Recently, metal based probes for phosphates have provided a successful strategy.<sup>5,6,10</sup> Among them, those that exhibit fluorescence enhancement in the presence of  $Zn^{2+}$  ion are highly attractive owing to its strong affinity for phosphates.<sup>11</sup> In particular, the phenanthroimidazole moiety exhibits electron withdrawing properties when linked with an electron donor (amino phenol) and rigidity associated electronic properties<sup>12</sup> provide us with a basic building block for the tuning of emissions of probes. However, phenanthroimidazole-based fluorophore has not been previously exploited in the development of metal based fluorescent PPi probes. In this context, herein we report a new phenanthroimidazole tagged Mannich base type fluorescent probe  $R-Zn^{2+}$  for PPi with the LOD of ppb level and its application in fluorescent detection of PPi released during DNA polymerase chain reaction (PCR).

Compound **R** was synthesized by refluxing of precursor compound PC-R with paraformaldehyde and di(2-picoly)amine in ethanol/ $CH_3COOH$  for 5 days (yield = 46%). The receptor  $R-Zn^{2+}$  (Scheme 1) was prepared by refluxing a methanolic solution

of  $Zn(NO_3)_2 \cdot 6H_2O$  and **R**. PC-R, **R** and the receptor  $R-Zn^{2+}$  were characterized by FTIR, multinuclear ( $^1H$ ,  $^{13}C$ ) NMR, ESI-MS and elemental analysis (Figs. S1–S8, ESI†). All the analyses are consistent with the proposed structural formula of the compounds.



Scheme 1 Synthesis of dizinc(II) probe ( $R-Zn^{2+}$ )

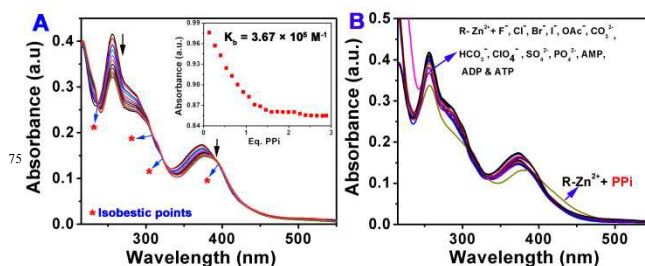


Fig. 1 Change in the initial absorbance spectrum of receptor  $R-Zn^{2+}$  ( $5 \mu M$ ) upon gradual increase in concentration of PPi ( $0-50 \mu M$ ) (inset shows the 1:1 binding isotherm at  $\lambda = 374 \text{ nm}$ ) (A) and by the presence of 1.0 equivalent of different anions (B), in 0.02 M HEPES at pH = 7.8.

The sensitivity of the receptor  $R-Zn^{2+}$  for various anions was studied by electronic, fluorescence and  $^{31}P$  NMR spectroscopy.

The titration studies were performed in 0.02 M HEPES (4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid) buffer at pH = 7.8. The absorption spectrum of  $\mathbf{R-Zn}^{2+}$  (5  $\mu\text{M}$ ) exhibits three bands at ( $\lambda_{\text{max}}$ , nm ( $\epsilon$ ,  $\text{M}^{-1} \text{cm}^{-1}$ )) 272 (86,000), 293 (58,000) and 374 (35,600) (Fig. 1A).

By incremental addition of the PPI (0–50  $\mu\text{M}$ ), the band of  $\mathbf{R-Zn}^{2+}$  at 374 nm shows a gradual reduction in the absorption intensity with a red shift (ca.  $\sim 5$  nm). The well-defined four isobestic points centered at  $\lambda = 238, 305, 322$  and 394 nm are consistent with an equilibrium between the receptor  $\mathbf{R-Zn}^{2+}$  and  $\mathbf{R-Zn}^{2+}\text{-PPI}$  in solution. The change in absorbance at 374 nm as a function of PPI concentration gave the best nonlinear fit for the 1:1 stoichiometry ( $K_b$ , binding constant of  $3.67 \times 10^5 \text{ M}^{-1}$ ). Notably, addition of other anions ( $\text{F}^-$ ,  $\text{Cl}^-$ ,  $\text{Br}^-$ ,  $\text{I}^-$ ,  $\text{CH}_3\text{COO}^-$ ,  $\text{HCO}_3^-$ ,  $\text{CO}_3^{2-}$ ,  $\text{N}_3^-$ ,  $\text{ClO}_4^-$ ,  $\text{SO}_4^{2-}$ , Pi, AMP (adenosine monophosphate), ADP (adenosine diphosphate) and ATP) did not show any significant change in the absorption spectrum of  $\mathbf{R-Zn}^{2+}$  (Fig. 1B). This clearly indicates that our receptor  $\mathbf{R-Zn}^{2+}$  possesses a remarkable binding selectivity for PPI in the ground state even in the presence of other anions.

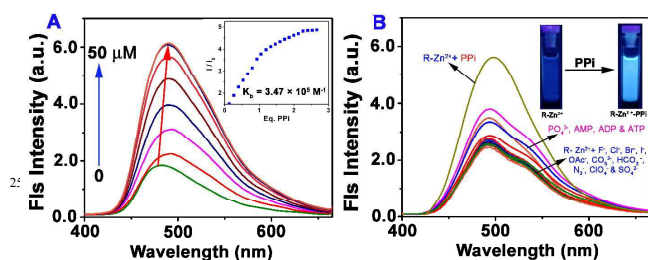


Fig. 2 Change in the initial fluorescence intensities of probe  $\mathbf{R-Zn}^{2+}$  (5  $\mu\text{M}$ ) upon gradual increase in concentration of PPI (0–50  $\mu\text{M}$ ) (inset shows the binding isotherm) (A) and by the presence of 1.0 equivalent of different anions (B), in 0.02 M HEPES at pH = 7.8.

The fluorescence spectrum of  $\mathbf{R-Zn}^{2+}$  (5  $\mu\text{M}$ ) shows a weak emission at 482 nm ( $\Phi = 0.16$ ) upon excitation at 365 nm. This was not affected upon addition of an excess of  $\text{CH}_3\text{COO}^-$ ,  $\text{CO}_3^{2-}$ ,  $\text{HCO}_3^-$ ,  $\text{SO}_4^{2-}$ , etc. However, for the phosphate analogues Pi, ADP and ATP, a considerable enhancement occurs. In contrast, PPI led to a red shift of the emission band to a longer wavelength at 495 nm ( $\Phi = 0.50$ ), which significantly enhanced ( $\sim 3.2$  fold increase) upon addition of  $\sim 1.0$  equivalent of PPI (Fig. 2). This enhancement of fluorescence intensity of  $\mathbf{R-Zn}^{2+}$  results from the selective CHEF (chelation enhanced fluorescence) effect<sup>13</sup> with PPI. The change in fluorescence emission with the PPI concentration (see stoichiometry plot of Fig. S9, ESI<sup>†</sup>) revealed the 1:1 stoichiometry between  $\mathbf{R-Zn}^{2+}$  and PPI with the calculated  $K_b$  of  $3.47 \times 10^5 \text{ M}^{-1}$ . This is higher than the  $K_b$  values of the phosphate analogues Pi, ADP and ATP with  $\mathbf{R-Zn}^{2+}$  ( $2.52 \times 10^4$ ,  $2.03 \times 10^4$  and  $2.11 \times 10^4 \text{ M}^{-1}$ , respectively) (Fig. S10, ESI<sup>†</sup>). ESI-MS (Fig. S11, ESI<sup>†</sup>) detected a species at  $m/z = 590.13$  corresponding to  $[\text{C}_{47}\text{H}_{55}\text{N}_8\text{O}_{16}\text{P}_2\text{Zn}_2]^{2-}$  ( $= [\mathbf{R-Zn}^{2+}\text{-PPI}(\text{H}_2\text{O})_8\text{-K}^+\text{-H}^+]^{2-}$ ), thus supporting the formation of a 1:1 complex. The chelation mode was further supported by  $^{31}\text{P}$  NMR spectroscopy. As seen in Fig. S12 (ESI<sup>†</sup>), both P atoms in PPI are magnetically equivalent and exhibit a single resonance at  $-6.40$  ppm. Upon binding with  $\mathbf{R-Zn}^{2+}$ , an upfield shift ( $\Delta\delta = 3.93$  ppm) was detected (Fig. 3A), indicating that  $\mathbf{R-Zn}^{2+}$  directly interacts with the phosphate sites.<sup>5d,e</sup>

To further prove the selectivity of the receptor  $\mathbf{R-Zn}^{2+}$  for PPI, we have performed the fluorescence titrations of  $\mathbf{R-Zn}^{2+}$  with other anions. Only PPI (Fig. 2B) showed a significant fluorescence enhancement (the other anions displayed weak fluorescence responses under identical conditions as those used with PPI). Hence,  $\mathbf{R-Zn}^{2+}$  can be considered a highly selective fluorescence probe for PPI in water. To validate the applicability of  $\mathbf{R-Zn}^{2+}$  as a selective probe for PPI, we performed a competitive fluorescence titration study with the other mentioned anions. The initial fluorescence intensity of  $\mathbf{R-Zn}^{2+}$  (Fig. 3B) in the presence of one equivalent of any of the other anions (green bars) increases substantially upon addition of one equivalent of PPI (blue bars), which further confirms the high selectivity of  $\mathbf{R-Zn}^{2+}$  for PPI in water even in the presence of those interfering anions. Although the phosphate analogues Pi, ADP and ATP considerably increased the fluorescence intensity, their binding affinity for  $\mathbf{R-Zn}^{2+}$  was not sufficient to enable the CHEF mechanism. In addition, the titration profile also reveals the LOD of  $9.1 \times 10^{-8} \text{ M}$  (0.25 ppm) for PPI (Fig. S13, ESI<sup>†</sup>), which is comparable to those of reported PPI probes (for a detailed comparison, see Table S1, ESI<sup>†</sup>).<sup>7, 14, 15a</sup> This observation means that  $\mathbf{R-Zn}^{2+}$  can even detect PPI at ppm concentrations in water. The sensitivity and selectivity of  $\mathbf{R-Zn}^{2+}$  toward PPI was further confirmed by time dependent fluorescence studies (Fig. S14, ESI<sup>†</sup>).

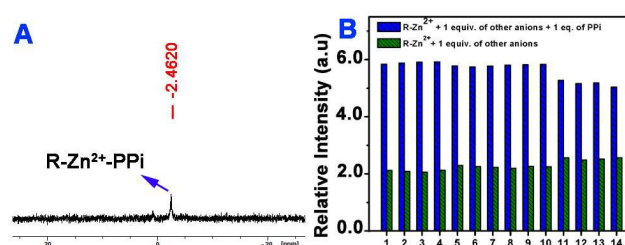
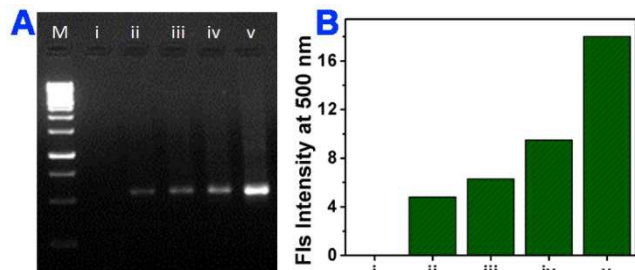


Fig. 3 (A)  $^{31}\text{P}$  NMR spectrum of the complex of PPI with one equivalent of  $\mathbf{R-Zn}^{2+}$  in  $\text{D}_2\text{O}$ . (B) Competitive selective binding affinity of probe  $\mathbf{R-Zn}^{2+}$  (5  $\mu\text{M}$ ) toward PPI in the presence of 1.0 equivalent of different anions in 0.02 M HEPES (pH = 7.8) (From 1 to 14:  $\text{F}^-$ ,  $\text{Cl}^-$ ,  $\text{Br}^-$ ,  $\text{I}^-$ ,  $\text{CH}_3\text{COO}^-$ ,  $\text{HCO}_3^-$ ,  $\text{CO}_3^{2-}$ ,  $\text{N}_3^-$ ,  $\text{ClO}_4^-$ ,  $\text{SO}_4^{2-}$ , Pi, AMP, ADP and ATP).

Pyrosequencing is a simple, rapid, and homogeneous phase detection technique to assess the amplified DNA after PCR.<sup>15</sup> It is based on the fluorescent detection of the PPI released from the deoxynucleotide triphosphates (dNTPs), when DNA is synthesized by the enzymatic action of DNA polymerase. Liquid phase reverse transcriptase PCR was performed with fungal mycelium oligo (dT)<sub>18</sub> primer to amplify the cDNA gene of *Pestalotiopsis microspore* (Taxol producing strain), and the PPI produced in specific cycle numbers was detected in solution with  $\mathbf{R-Zn}^{2+}$ . It is important that the fluorescent probe should specifically diagnose the released PPI in the presence of related anionic nucleotide analogues N (Fig. S15, ESI<sup>†</sup>). We have now applied  $\mathbf{R-Zn}^{2+}$  as a PPI fluorescent probe, since it can detect traces of PPI in presence of a high ATP excess. The formation of DNA of the same molecular weight is shown by gel electrophoresis (Fig. 4A), the band intensity being proportional to the concentration of PPI released from PCR and detected at 500 nm (Fig. 4B). This agrees with the hypothesis that the extent of the fluorescence changes in  $\mathbf{R-Zn}^{2+}$  is proportional to both the concentration of PPI released during PCR and the degree of DNA polymerization. This technique may be a simple and rapid alternative for estimating the products of PCR and the probe  $\mathbf{R-Zn}^{2+}$ .

$\text{Zn}^{2+}$  has also a potential application in the new generation of DNA sequencings in view of its selectivity and sensitivity.



**Fig. 4** (A) Gel electrophoresis of finished PCR mixtures. M: shown as DNA marker. (B) Fluorescence intensity of probe  $\text{R-Zn}^{2+}$  at 500 nm upon addition of the finished PCR product mixture. i: 10  $\mu\text{L}$  of finished PCR product mixture performed without template DNA; 10  $\mu\text{L}$  of finished PCR product mixture performed with template DNA after ii: 29 cycles; iii: 30 cycles; iv: 31 cycles; v: 32 cycles.

In summary, we have designed and synthesized a phenanthroimidazole based probe  $\text{R-Zn}^{2+}$  which displays a highly selective and sensitive fluorescence response for PPI in comparison with the related phosphates Pi, AMP, ADP, ATP and other anions in water. Its remarkable LOD (0.25 ppm) should allow the detection of trace quantities of PPI in biological and environmental samples. Those features are favourable to the use of that probe for fluorescent recognition of PPI and confirmation of amplified DNA in the PCR products.

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## Notes and references

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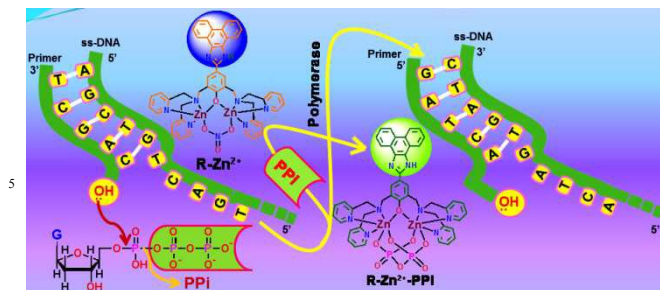
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†Electronic Supplementary Information (ESI) available: Experimental procedures, NMR, ESI MS spectral data of the compounds, sensor studies and PPI sensing mechanism of  $\text{R-Zn}^{2+}$ . See DOI: 10.1039/b000000x/

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## TOC



Phenanthroimidazole tagged Mannich base type dizinc(II) complex as a unique, very sensitive and efficient fluorescent probe for pyrophosphate ion (PPi) in water with a very low detection limit, also being used to detect PPi released from DNA polymerization chain reaction.