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A Highly Selective Pyrophosphate Sensor Based on ESIPT Turn-On in Water

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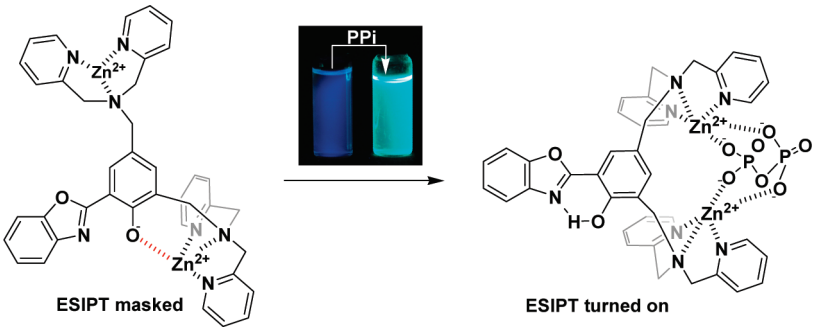
ABSTRACT



Pyrophosphate (PPi) is a biologically important target. A binuclear system 3•2Zn is found to selectively recognize PPi, leading to a ratiometric fluorescent sensor at pH 7.4 in water. The binding event triggered a large fluorescence response (~100 nm bathochromic shift) by turning on the excited state intramolecular proton transfer (ESIPT). Detection of PPi released from a PCR experiment indicated that this new probe could be a useful tool in bioanalytical applications.

Pyrophosphate ($P_2O_7^{4-}$, PPi) is a biologically significant anion which is involved in many cellular processes,[1] such as cellular ATP hydrolysis, DNA and RNA polymerizations, and enzymatic reactions. It has been reported that abnormal PPi levels can lead to vascular calcification resulting in severe medical conditions.[2] Since Czarnik's pioneering work in fluorescent sensing of PPi in 1994 by using a polyamine-attached anthracene derivative in 100% aqueous solution,[3] considerable effort has been made to develop chemosensors for the optical detection of PPi.[4] In order to achieve selective binding for PPi, the current strategy utilizes a binuclear metal[5a–j] complex in conjunction with bis(2-pyridylmethyl)amine (DPA) ligands as shown in **1**.

An example of **1** (R = —N=N—Ar)[5a] illustrates the selective binding of PPi to give a *non*-fluorescent **2** and shows a color response (λ_{max} red shift by 48 nm). Despite strong interest in developing fluorescent PPi sensors,[5d,h] the PPi binding has induced a relatively small red shift in fluorescence signal (~11 nm from a fluorescein-derivative[5d] or ~20 nm from a coumarin derivative).[5h] Lack of significant fluorescence signal shift lowers the sensitivity and hampers the reliable fluorescent detection for PPi. In

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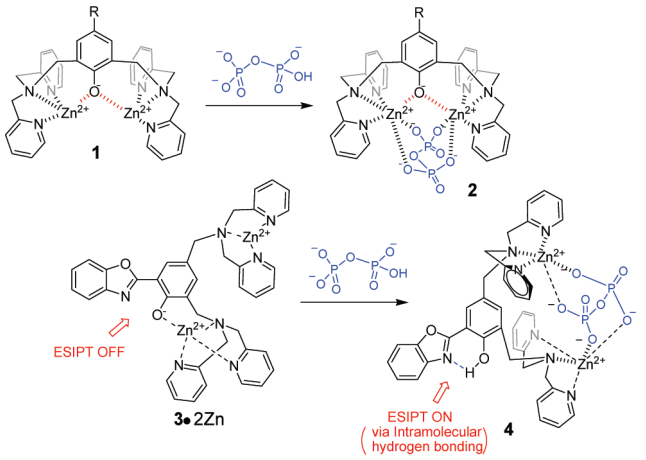
Scheme 1. Molecular Design of PPi Sensor with ESIPT



the current PPi sensing systems (e.g., from **1** to **2**), the PPi only induces a minor structural change on the chromophore, thereby resulting in the limited spectral shift.

Intramolecular hydrogen bonding in 2-(2-hydroxyphenyl)-1,3-benzoxazole (HBO) derivatives is known to exhibit excited state intramolecular proton transfer (ESIPT),[6] which gives a large Stokes shift (100−200 nm). Extending our general interest in exploring the analyte binding-enabled ESIPT,[7] we now report the PPi sensor **3**·2Zn, in which the ESIPT is used as a switching mechanism in a buffered aqueous solution (Scheme 1). In the PPi sensor **3**·2Zn, the phenoxide "oxygen" is used to chelate the Zn^{2+} center to disable ESIPT. The analyte recognition requires PPi to simultaneously connect to the two Zn^{2+} sites in **4**, which moves the Zn^{2+} away to free the phenol moiety for ESIPT. The well-defined chemical event in the molecular design leads to a highly selective probe for PPi, with fluorescence changing from blue to green (corresponding to the bathochromic shift of ∼100 nm, Figure 1).

The sensor **3**•2Zn was synthesized from 2-(2′-hydroxyphenyl)benzoxazole **5** (Scheme 2). The dialdehyde intermediate **6** was obtained by a dual-Duff reaction, which was further transformed into **3** by a reductive amination to install the two bis(2-pyridylmethyl)amino groups. The dinuclear Zn^{2+} complex **3**·2Zn was formed by the addition of a methanolic solution of **3** to an aqueous solution of 2.0 equiv of $Zn(NO_3)_2$. The overall yield is 55% from **5**. For comparison, a mononuclear Zn^{2+} complex **8**•Zn was synthesized in a similar route in an overall yield of 49% from 2-hydroxy-5-methylbenzaldehyde **7**. The experimental details and characterization data are given in the Supporting Information (SI).

Figure 1 shows the effect of anions (sodium salts) on the fluorescence spectrum of **3**•2Zn in a buffered aqueous

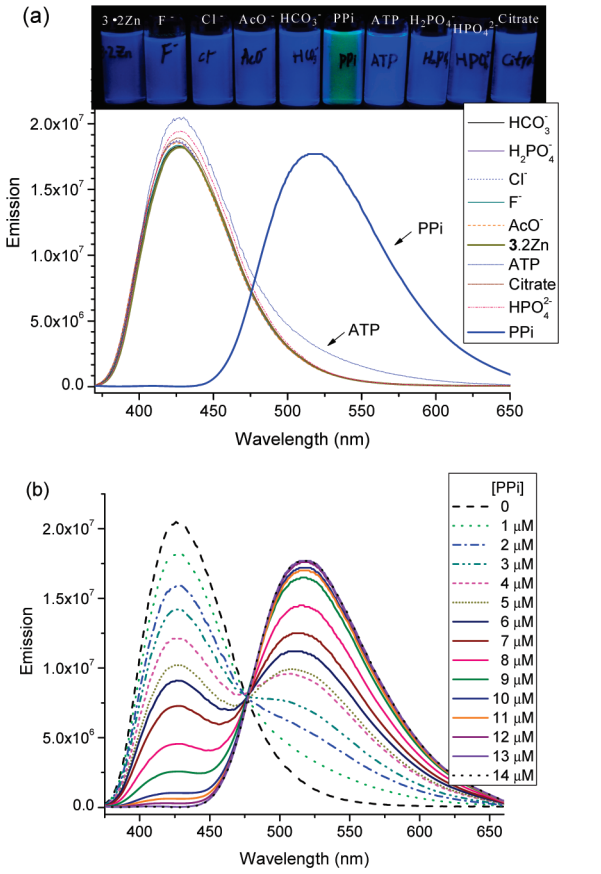
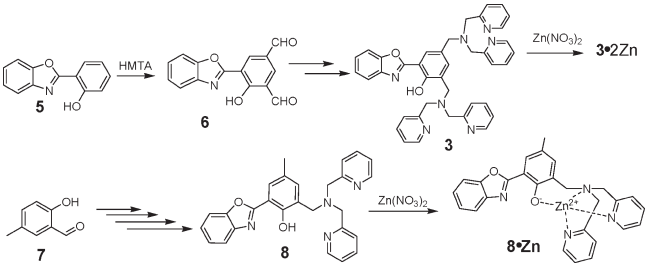


Figure 1. (a) FL spectra of sensor **3**·2Zn (12 μM) in aqueous solution of 10 mM HEPES buffer (pH 7.4) at 25 °C in the presence of various anions (600 μM) and their fluorescent images (irradiated at 365 nm). (b) Change in fluorescence emission of **3**·2Zn (12 μM) upon addition of PPi (sodium salt) in an aqueous solution of HEPES buffer (10 mM, pH 7.4) at 25 °C.

Scheme 2. Synthetic Strategy for Sensor **3**•2Zn and **8**•Zn



solution (pH = 7.4). In the absence of an anion guest, senor **3**•2Zn gives emission at 420 nm. This fluorescent emission was not changed upon addition of excessive HPO_4^{2-}, $CH_3CO_2^-$, citrate, etc. In contrast, the PPi anion caused the emission band to be shifted to a longer wavelength (at 518 nm), attributed to the *keto* emission arising from ESIPT (SI, Scheme S1). Although the related phosphate analogues ATP slightly increased the fluorescence

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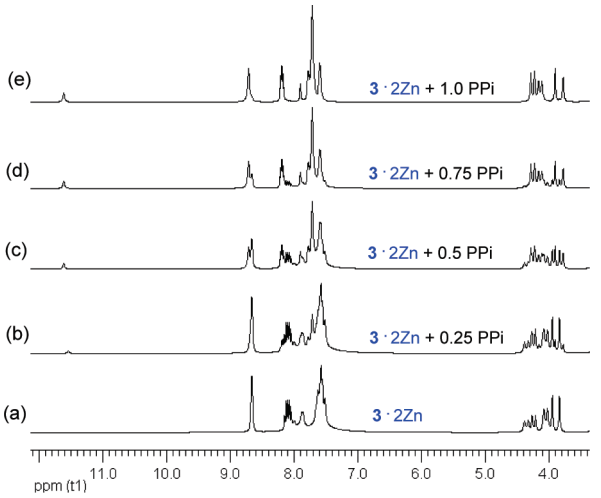
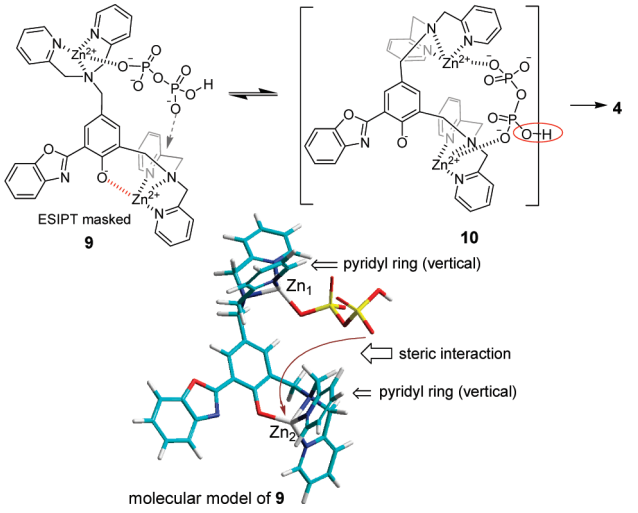


Figure 2. ^{1}H NMR spectra of **3**•2Zn (1.5 mM) with various molar ratios of hydrogen pyrophosphate ($Na_3HP_2O_7$, HPPi)[8] in DMSO-d_6.

Scheme 3. Proposed ESIPT Turn on Mechanism[a]



[a] For clarity, the NO_3^- ligands to zinc were omitted. Molecular modeling of **9** (optimized by using AM1 setting on HyperChem) shows that two vertical pyridyl rings on the top of plane form a narrow entrance gate.

intensity, its binding with **3**•2Zn was not sufficiently strong to enable the ESIPT. Job's plot (SI, Figure S3) revealed that the complex had a 1:1 stoichiometry for **3**•2Zn and PPi. The formation of a 1:1 complex was further supported by electrospray mass spectrometry (ES-MS) data (SI, Figures S5 and S6), which detected the complex at m/z = 936 corresponding to $[C_{39}H_{36}N_7O_9P_2Zn_2]^+$ (= [**3**•2Zn + PPi + H^+]$^+$) and a peak at m/z = 934 corresponding to the deprotonated phenoxide, $[C_{39}H_{35}N_7O_9P_2Zn_2]^-$ (= [**3**•2Zn + PPi − H^+]$^-$).

Upon titration of **3**•2Zn with hydrogen pyrophosphate,[8] the ^{1}H NMR spectra (Figure 2) revealed a new peak at 11.6 ppm corresponding to the intramolecular H-bonding between the phenolic proton and benzoxazole nitrogen.[9] The intensity of this peak increased with the amount of HPPi. The result indicated that the phenoxide oxygen was released from the metal-binding by the cooperative coordination of PPi with the two Zn^{2+} ions as shown in Scheme 1.

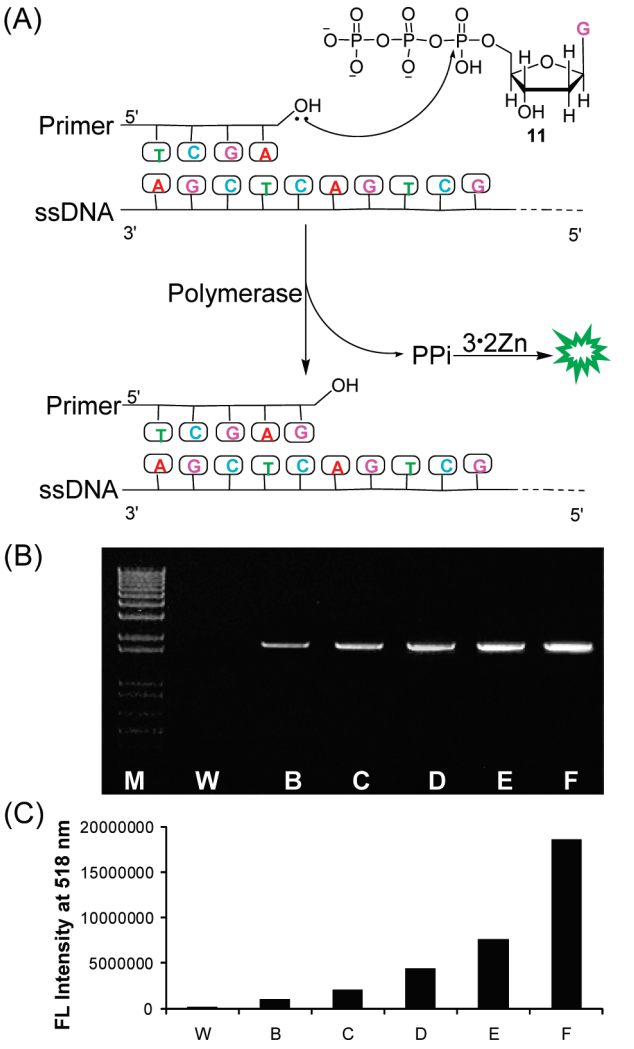


Figure 3. (A) Mechanism of sensing PPi released from PCR by **3**•2Zn. (B) Gel electrophoresis of finished PCR mixtures. M: shown as DNA marker. (C) Fluorescence intensity of sensor **3**·2Zn at 518 nm upon addition of the finished PCR product mixture. W: 10 μL of finished PCR product mixture performed without template DNA; 10 μL of finished PCR product mixture performed with template DNA after B: 21 cycles; C: 22 cycles; D: 23 cycles; E: 24 cycles; F: 25 cycles.

(8) The four pK_a's of pyrophosphoric acid ($H_4P_2O_7$) were reported to be 0.9, 2.0, 6.6, 9.4 (McElroy, W.D.; Glass, B. *Phosphorus Metabolism*; Johns Hopkins University Press: Baltimore, 1951; Vol. I). At pH 7.4, an aqueous solution of pyrophosphoric acid consists of 85.6% HA^{3-}, 13.6% H_2A^{2-}, 0.7% A^{4-}.

(9) The chemical shift of the intramolecular H-bonding in the HBO system is typically around 11 ppm. Chen, W.-H.; Pang, Y. *Tetrahedron Lett.* **2009**, *50*, 6680.

To further understand the role of the binuclear Zn(II)... Zn(II) structure on the observed PPi selectivity, mononuclear **8**•Zn was prepared and titrated with PPi under the same conditions. Addition of PPi increased the fluorescence intensity at 427 nm without inducing a notable bathochromic shift (SI, Figure S1). The result indicated that no ESIPT was induced from the **8**•Zn complex. In other words, PPi was bound to the Zn^{2+} in the mononuclear complex without breaking the existing Zn−O bond. From the fluorescence titration experiments, the association constants of **3**•2Zn with PPi and **8**•Zn with PPi were calculated to be 9.2×10^7 and 1.8×10^4 M^{-1} respectively.[10] It therefore can be concluded that the ESIPT turn-on in **3**·2Zn required the analyte to simultaneously bind to two Zn^{2+} sites. It was assumed that interaction of **3**•2Zn with the PPi anion initially led to **9** (Scheme 3). The negative charged oxygen at the other end of PPi then reached out to the second Zn^{2+} site to form **10**. Molecular modeling of **9** showed that the Zn_1 and Zn_2 centers were separated by 7.35 Å, which was beyond the distance a monoanion such as HCO_3^{2-} and $H_2PO_4^-$ can bridge. As seen from the model of **9**, the pyridyl ring at the second zinc center (labeled Zn_2) acted as a gate to allow the less hindered phosphate anion to pass. In contrast, the bulky tail of ATP would have increased steric interaction with the pyridyl ring at the gate, which prevents the associated phosphate anion from entering into the cavity to bind to the second Zn^{2+} site. In other words, the unique steric interaction posed by the pyridylmethyl group plays an important role in the sensor's ability to differentiate PPi from the structural analogue ATP.

Recently, pyrosequencing,[11] a revolutionary DNA sequencing technique based on the "sequencing by synthesis" principle, is coming into fashion.[12] The pyrosequencing technique relies on the enzymatic detection of the pyrophosphate released during the DNA polymerase chain reaction (PCR). The application requires the probe to specifically recognize the released PPi, in the presence of structurally similar anionic nucleotides **11** (Figure 3). To demonstrate the applicability, sensor **3**•2Zn was used and found to be a simple and rapid method to fluorescently detect the PPi released from dNTPs in a PCR. Gel electrophoresis revealed the production of DNA of the same molecular weight (Figure 3b), where the band intensity was proportional to the amount of PPi released from PCR and subsequently detected by the fluorescence intensity at 518 nm (Figure 3c). This result showed that the sensor **3**•2Zn had potential application in the new generation of DNA sequencings by virtue of its selectivity and sensivitity.

In summary, we have developed a new ratiometric sensor based on a unique ESIPT turn-on mechanism. The two DPA-Zn^{2+} groups in the sensor **3**•2Zn, which were located at a suitable distance, create a strong binding environment to selectively recognize PPi over structurally similar phosphate ATP and other anions. Utilization of this molecular recognition event to trigger the ESIPT results in a large fluorescent bathochromic shift (~100 nm). A large fluorescence response, in addition to its excellent selectivity toward PPi, points to the probe molecule's potential use for biochemical and analytical applications.

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Supporting Information Available. Experimental procedure and spectral data for all new compounds, optical spectra of **3**•2Zn and **8**•Zn. This material is available free of charge via the Internet at http://pubs.acs.org.

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