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PAPER

Highly selective colorimetric sensing pyrophosphate in water by a NBD-phenoxo-bridged dinuclear Zn(II) complex†

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A novel NBD-phenoxo-bridged dinuclear Zn(II) complex is found to be an effective colorimetric sensor for pyrophosphate (PPi) in pure aqueous solution over a wide pH range. This sensor shows high binding affinity ($K_a \approx 3 \times 10^8$ M^{-1}) and high selectivity for PPi, and can be also used to assay the activity of pyrophosphatase in real time.

Introduction

Much attention has been given in recent years to the development of receptors and sensors for anions due to their important roles in various chemical and biological processes.[1] Among them, pyrophosphate ($P_2O_7^{4-}$, PPi) is one of the most popular targets for recognition studies because it plays very important roles in several biological processes.[2] PPi is a product of hydrolysis of ATP and other nucleotide triphosphates under cellular conditions, and is vital for energy transduction and the control of metabolic processes in organisms *via* its participation in various enzymatic reactions.[3] The detection of the PPi concentration level has been examined as a real-time DNA sequencing method[4] and recently has become an important issue in cancer research.[5] The level of PPi is also related to several diseases, for example, a lack of PPi can lead to calcium deposits in arteries – called Mönckeberg's arteriosclerosis (MA)[6] and too much PPi can cause calcium pyrophosphate deposition disease (CPDD).[7] PPi is also the most common cause of cultural eutrophication as an environment pollutant.[8] Therefore, the specific recognition, detection and sensing of PPi in aqueous solution is of widespread significance.[9]

Due to the strong hydration of anions in water and the structural similarity of the phosphate anion family, the creation of effective PPi sensors in aqueous solution is challenging. It requires both high affinity for PPi in water and, importantly, high selectivity for PPi over other related analogues such as inorganic phosphate (Pi), ADP, AMP, and particularly the equally charged ATP tetraanion, as well as the ability to convert PPi recognition into a fluorescent or colorimetric signal. Hence, during the past decade, only a few examples of effective fluorescent and colorimetric sensors for PPi in 100% aqueous solutions have been reported.[9–12] Pioneered by the work in Czarnik's group,[10] most effort has been devoted to the development of fluorescence chemosensors for PPi in aqueous solution.[11] Among them, the utilization of metal complexes, particularly zinc complexes with a bis-(2-pyridylmethyl)amine (DPA) unit as a binding site for PPi, has been found to be the most successful strategy.[11b–i] Indicator-displacement assays (IDA) have also been used to detect PPi when the metal complex lacks an available fluorophore or chromophore.[13] Although fluorescent sensors for PPi have been developed most successfully to date,[11,12] effective colorimetric sensors for PPi in pure water are rather rare.[14–18] The latter has an outstanding advantage – it can be easily observed and determined by naked eye so that the measurements by analytical instruments may be minimized or even eliminated.

Here we report a new NBD-phenoxo-bridged dinuclear Zn(II) complex **1·2Zn** (Fig. 1), which is an effective colorimetric sensor for PPi, not only with high sensitivity in pure aqueous solution of a wide pH range, but also with high selectivity for PPi over many other anions including ATP and Pi.

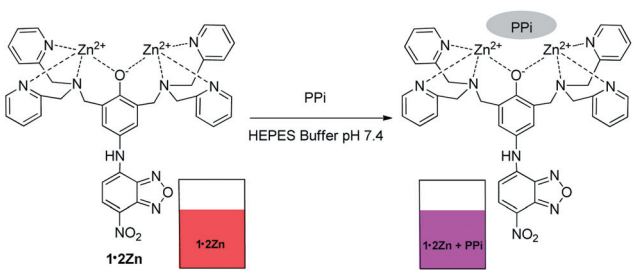

Fig. 1 Sensor **1·2Zn** and colorimetric sensing of PPi.

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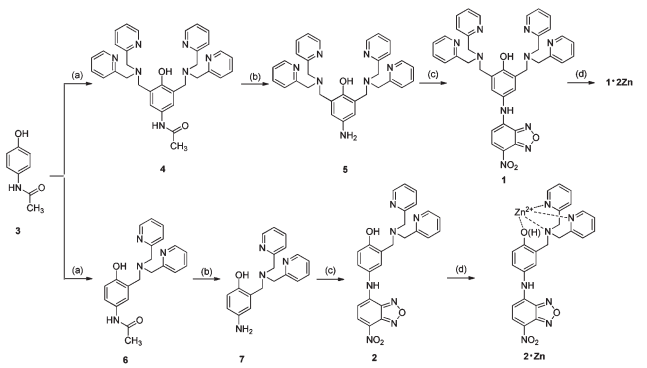
†Electronic supplementary information (ESI) available: Job's plot for PPi and ATP with **1·2Zn**, sensing selectivity of sensor **1·2Zn** for PPi over Pi, ADP and ATP, sensing of PPi at different pH values, studies of **2·Zn**, some of the NMR spectra. See DOI: 10.1039/c2ob25617g

Results and discussion

Sensor **1·2Zn** was prepared from 4-acetamidophenol in four steps (Scheme 1). The chromophore 7-nitrobenz-2-oxa-1,3-diazole (NBD) in sensor **1·2Zn** was selected because it can be easily introduced and it absorbs at long wavelengths (~500 nm),[19] to offer a sensor which does not have much background absorption to compete with. **1·2Zn** can be easily formed by mixing ligand **1** with 2 equiv of zinc nitrate in methanol and it dissolves readily in pure water forming a red solution. As a control, mononuclear complex **2·Zn** was also prepared in a similar manner (Scheme 1).

We first tested whether the red color of the **1·2Zn** solution can be affected by addition of anions (sodium salts). When one equiv. of PPi was added to sensor **1·2Zn** (35 μM) in an aqueous solution of HEPES buffer (50 mM, pH 7.4, HEPES = 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid), the color of the sensor solution changes immediately from red to purple (Fig. 2). In contrast, no color changes upon addition of other inorganic anions such as either monovalent anions $H_2PO_4^-$, F^-, Cl^-, Br^-, $CH_3CO_2^-$, NO_3^-, HCO_3^-, ClO_4^- N_3^- or di/trivalent anions HPO_4^{2-} (Pi), SO_4^{2-}, $S_2O_7^{2-}$, $C_2O_4^{2-}$, citrate, PO_4^{3-} even up to an excess of 1000 equivalents. Other organic phosphate anions such as AMP, ADP, ATP, phenyl phosphate (PhPi), 4-nitrophenyl phosphate (NPhPi) were also tested. Only the addition of excess ATP induces a slight color change. As a control, mononuclear **2·Zn** does not show any observable color changes even upon the addition of a large excess of PPi and other anions (Fig. S4, ESI†). These results indicate that only the dinuclear complex **1·2Zn** can be used as a colorimetric sensor that shows a high selective coloration for PPi under physiological conditions.

To shed further light on this sensor, the effect of anions on the absorption spectrum of sensor **1·2Zn** were then examined in an aqueous HEPES buffer (50 mM, pH 7.4) at 25 °C. Under these conditions, sensor **1·2Zn** (35 μM) showed a maximum absorption band centred at 501 nm with an extinction coefficient of 22 700 M^{-1} cm^{-1}. As shown in Fig. 3a, upon addition of PPi in increasing amounts, the peak at 501 nm decreases and a new peak appears at 526 nm. The 25 nm red shift is consistent with the color change observed from red to purple. The absorbance at 600 nm increases linearly until it reaches a plateau with the addition of more than one equiv. of PPi (inset in Fig. 3a), which indicates a tight 1 : 1 binding between **1·2Zn** and PPi at these concentrations. This 1 : 1 binding stoichiometry was also confirmed by Job's plot (Fig. S1, ESI†). Unlike the large 25 nm



Scheme 1 Synthesis scheme for **1·2Zn** and **2·Zn**. *Reagents and conditions*: (a) dipicolylamine (DPA), paraformaldehyde in C_2H_5OH, 80 °C. (b) aq. HCl, 100 °C. (c) 4-Chloro-7-nitro-2,1,3-benzoxadiazole (NBD-Cl), $NaHCO_3$, MeOH, rt. (d) $Zn(NO_3)_2$, H_2O, MeOH.

Fig. 2 Color changes of sensor **1·2Zn** in 50 mM aqueous HEPES buffer solution (pH 7.4), [**1·2Zn**] = 35 μM, [PPi] = 35 μM, [other anions] = 175 μM. Anions from left to right: none, AMP, ADP, ATP, PO_4^{3-}, HPO_4^{2-} (Pi), $H_2PO_4^-$, PhPi, NPhPi, PPi, F^-, Cl^-, Br^-, I^-, NO_3^-, SO_4^{2-}, HCO_3^-, $CH_3CO_2^-$, citrate, N_3^-, ClO_4^-, $S_2O_7^{2-}$, $C_2O_4^{2-}$.

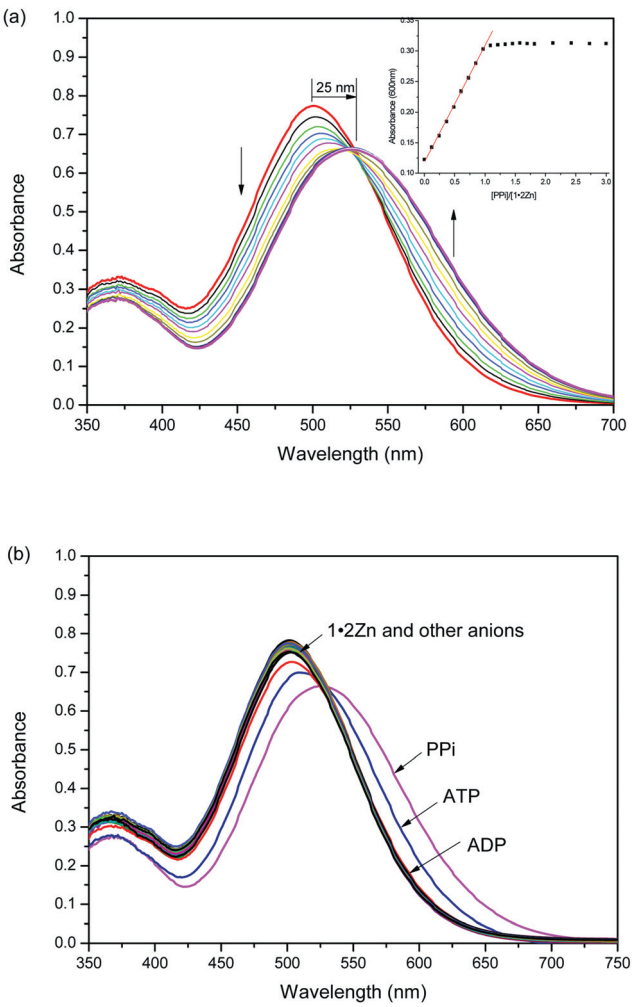


Fig. 3 (a) UV-vis spectra changes of sensor **1·2Zn** (35 μM) upon addition 0–3 equiv of PPi (sodium salt). Insert: the absorbance changes at 600 nm upon addition of PPi. (b) UV-vis spectra of sensor **1·2Zn** (35 μM) in the presence of various anions (35 μM). All the spectra were measured in pure aqueous solution of 50 mM HEPES buffer (pH 7.4) at 25 °C. Under these conditions, sensor **1·2Zn** is saturated with PPi (one equiv.) and ATP (one equiv.), and can be saturated with ADP (more than five equiv.) with a 4 nm red shift of its λ_{max}.

red shift observed with PPi, only a relatively small red shift (10 nm) is observed with ATP upon complexation, which is why ATP only induces a slight color change. The spectral changes and the degrees of red shift (0–4 nm) caused by other anions are almost negligible, so no color changes can be observed in these systems (Fig. 2 and 3b). The different degrees of red shift induced by PPi and other anions can be explained by the degree of electron donation of the phenolate oxygen atom into the phenyl ring. Only when the most charged PPi tightly binds to **1·2Zn** the bond between the phenolate oxygen atom and the two Zn^{2+} ions sufficiently weakened to leave a significantly more-negatively charged phenolate oxygen atom. This donates more electron density into the phenyl ring, and as a result, induces a large red shift. The red shift induced by PPi was also observed in Hong's elegant naphthalene-DPA based fluorescent[11b] and azaphenol-DPA based colorimetric[14] sensors, but both of them absorb at much shorter wavelengths. The red shift of the former sensor occurs outside the visible region and the latter requires higher concentration (60 μM) for naked eye detection of PPi.[20] The different response relative to the similarly charged ATP can be rationalized in terms of charge density. In PPi, both phosphoryl units carry two negative charges and can bind to both Zn^{2+} ions, whereas in ATP the same charge is distributed across three phosphoryl units. Hence, the same binding mode, involving four oxygen atoms with lower charge density, has less effect on the Zn^{2+}-ligand binding. It seems likely that an alternate binding mode involving all the phosphoryl groups is sterically precluded, but even in this case the α and β phosphoryl groups will be weaker donors than the γ and PPi phosphoryl oxygens.

To further understand this, as well as the mode of complexation, ^{31}P NMR spectroscopy studies were undertaken. As shown in Fig. 4a, both P atoms in PPi are magnetically equivalent and show a single signal at −6.5 ppm. However, on binding with **1·2Zn**, significant upfield shifts ($\Delta\delta$ = 5.30 ppm) were observed for the ^{31}P signal. The presence of a single ^{31}P signal suggests that both P atoms in the metal binded PPi are still magnetically equivalent and therefore the two sets of oxygen anions on each P atom of PPi are equally bound to the binuclear zinc complex. In the case of ATP binding with **1·2Zn**, upfield shifts for the three phosphorous signals in ATP were also found, but their shift degrees are quite different. The signals for β and γ phosphorous are upfield shifted by 1.48 and 0.77 ppm, respectively. In contrast, much smaller upfield shift for α phosphorous signal ($\Delta\delta$ = 0.15 ppm) was observed, which indicates the α phosphorous centre is not interacting (or has very week interactions) with the zinc centres of **1·2Zn**. Therefore, the binding force of ATP with **1·2Zn** mainly come from the interactions of the oxygen anions on β and γ phosphoryl groups of ATP with the metal centres of **1·2Zn**. Based on our ^{31}P NMR studies and the crystal structure of the PPi complex reported by Hong *et al.*,[14] the proposed binding modes for **1·2Zn** with PPi and ATP are shown in Fig. 4b.

In biological systems, PPi is released in the presence of ATP and other phosphates, so good sensors for PPi in water must show high selectivity for PPi over other highly anionic phosphates such as ATP and HPO_4^{2-} (Pi) as well as high affinity. Since this colorimetric sensor is based on the chromophore of nonfluorescent NBD-phenol, the detection limitation of this kind

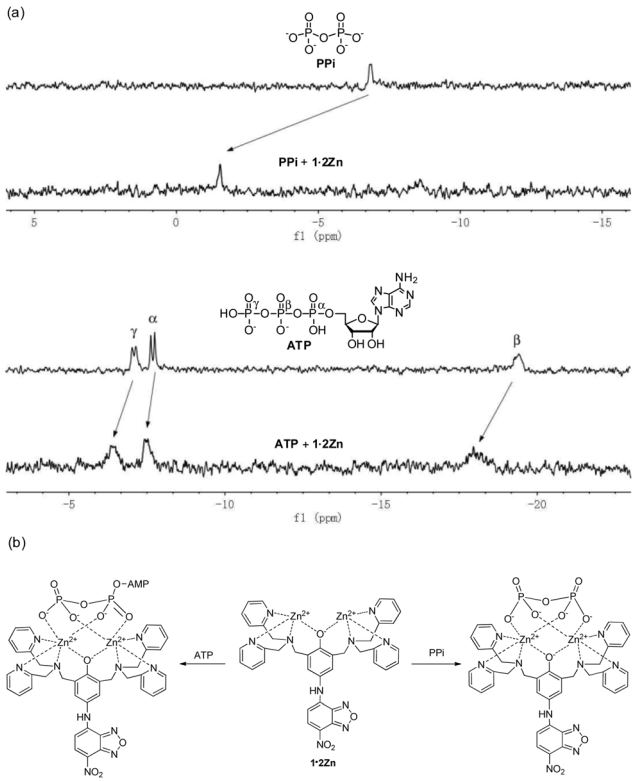


Fig. 4 (a) ^{31}P NMR spectra of PPi (4 mM) and ATP (4 mM) and with one equiv. of sensor **1·2Zn** in H_2O. (b) Proposed binding modes for **1·2Zn** with PPi and ATP.

Table 1 Apparent association constant (K_a) of sensor **1·2Zn** for anions PPi, ATP, ADP, AMP, HPO_4^{2-} and $H_2PO_4^-$ in an aqueous HEPES buffer (50 mM, pH 7.4) at 25 °C[a]

Anion	K_a (M^{-1})	Anion	K_a (M^{-1})
PPi	$(2.9 \pm 0.3) \times 10^8$	AMP	$(7.2 \pm 0.7) \times 10^4$
ATP	$(3.7 \pm 0.4) \times 10^6$	HPO_4^{2-}	$(2.3 \pm 0.2) \times 10^4$
ADP	$(4.6 \pm 0.4) \times 10^5$	$H_2PO_4^-$	$(2.1 \pm 0.2) \times 10^4$

[a] All anions were added as sodium salts. K_a for PPi and ATP were determined by competitive binding in the presence of 1000 fold excess of HPO_4^{2-}. For other anions, K_a was calculated by fitting the curve of absorbance changes against phosphate concentration *via* UV-vis titration.[21]

of sensor system would be low relative to the fluorescent chemosensors. In this case, achieving high-affinity binding is particularly important for improving detection limits. As shown in Table 1, **1·2Zn** indeed showed the strongest binding for PPi over other phosphates. The apparent association constant (K_a) value for the complexation of PPi with **1·2Zn** was determined to be $(2.9 \pm 0.3) \times 10^8$ M^{-1} in an aqueous HEPES buffer (50 mM, pH 7.4) at 25 °C, which is about 80-fold, 630-fold, 4000-fold and over 10 000-fold higher than for ATP, ADP, AMP and HPO_4^{2-} binding with **1·2Zn**, respectively. This nearly nanomolar affinity and the big differences in the affinity values between phosphate anions not only mean that **1·2Zn** can detect PPi at sub-micromolar concentrations in water, but also can detect PPi in the presence of a large excess of other phosphates,

even with the same charge. Indeed, an obvious color change from red to purple was also observed when PPi was titrated into the sensor **1·2Zn** solution in the presence of 1000 equiv. HPO_4^{2-}, 100 equiv. ADP or 50 equiv. ATP (Fig. S2, ESI†), thus making sensor **1·2Zn** a good candidate for bioanalytical applications. The same arguments presented above concerning the degree of red shift also apply for explaining why stronger binding is obtained for **1·2Zn** with PPi compared to ATP.[11b] To the best of our knowledge, our system is the first colorimetric sensor that not only has nanomolar affinity for PPi in pure water solutions, but also can effectively discriminate PPi from both Pi and ATP.[22]

We also tested the effect of pH on PPi sensing to evaluate the functional pH ranges of the sensor. Over the pH range 5.5–10.0, addition of PPi causes obvious color changes with 18–25 nm red shift of the maximum absorption band of **1·2Zn**, and these changes are still evident in the presence of a wide range of competing anions (Fig. S3, ESI†). This means **1·2Zn** works over a wide pH range, and even if the external pH is disturbed, **1·2Zn** still can detect PPi in the presence of various anions.

To demonstrate the potential of sensor **1·2Zn** in a practical application, a real-time assay was constructed to monitor the activity of inorganic pyrophosphatase (PPase). Inorganic PPase is essential in many important biosynthetic reactions and catalyses the hydrolysis of PPi into phosphate.[23] Based on our results, we thought sensor **1·2Zn** may provide an easy way to monitor the activity of inorganic PPase, even simply by observation of color change. Thus, the activity of inorganic PPase was tested in a real-time assay using an aqueous HEPES buffer containing 50 μM **1·2Zn** and 50 μM PPi. The reactions were monitored at 600 nm as a function of time immediately after addition of the given units of inorganic PPase. As shown in Fig. 5a, decays at 600 nm were observed over time after the addition of PPase, and the reaction accelerates with an increase in concentration of PPase. More importantly, as shown in Fig. 5b, this assay was accompanied by an obvious color change of the reaction mixture from purple to red, which means **1·2Zn** can be used to detect whether a given inorganic PPase sample is active or not without using any analytical instruments. Fig. 5c shows scanning kinetics of the assay in the presence of 0.2 units of inorganic PPase. Clearly, the UV-vis spectra changes of the PPase catalysed reaction in the presence of **1·2Zn** are just opposite to that of the titration experiments showed above in Fig. 3a. Therefore, we proved that **1·2Zn** can be used as a simple and convenient chemosensor for real-time probing the activity of inorganic PPase.

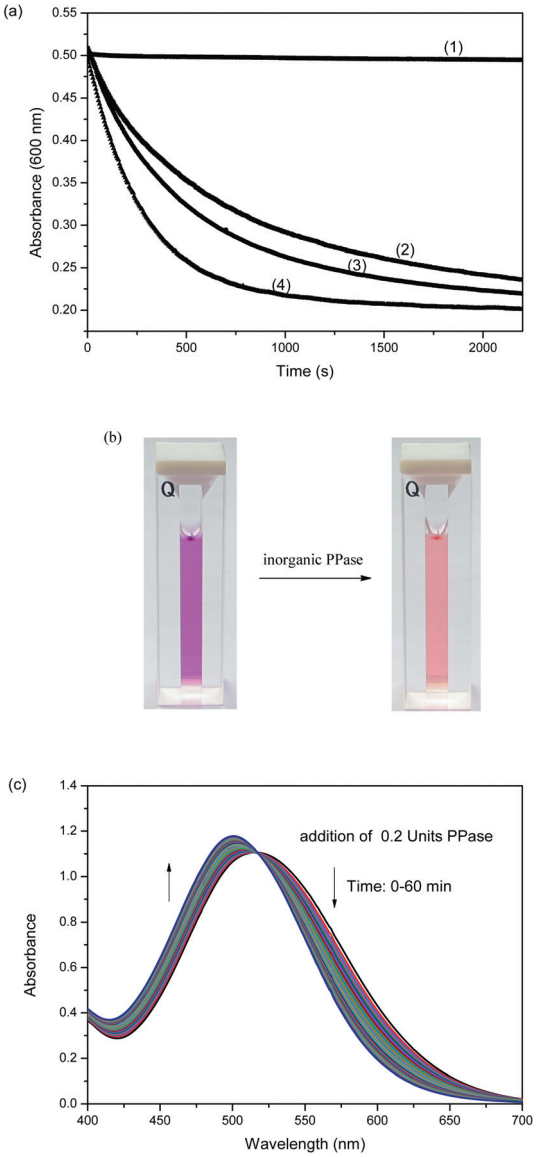


Fig. 5 (a) Kinetics of real-time monitoring PPi (50 μM) hydrolysis catalyzed by inorganic PPase in the presence of 50 μM **1·2Zn**. Reactions are monitored at 600 nm. Concentrations of inorganic PPase: (1) 0 units; (2) 0.1 units; (3) 0.2 units; (4) 0.4 units. (b) Color changes of the reaction catalysed by PPase. (c) Scanning kinetics of real-time monitoring PPi (50 μM) hydrolysis catalyzed by 0.2 units inorganic PPase. The UV-vis spectra were recorded every minute. All the reactions were recorded in an aqueous HEPES buffer (50 mM, pH 7.5) at 25 °C.

Experimental

General

Starting materials were purchased from commercial suppliers and were used without further purification. All solvents were purified by the most used methods before use. All solutions and buffers were prepared with using distilled water that had been passed through a Millipore-Q ultrapurification system. Buffers used for different pHs were 2-(*N*-morpholino)ethanesulfonic acid (MES), *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethane-sulfonic acid) (HEPES), 2-(*N*-cyclohexylamino)ethanesulfonic acid (CHES) and 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS). TLC analysis was performed using precoated plates. Column chromatography was performed using silica gel (200–300 mesh) using eluents in the indicated v : v ratio. IR spectra were recorded on a Perkin-Elmer Spectrum BX FT-IR spectrophotometer as KBr pellets and were reported in cm^{-1}. NMR spectra were measured on Varian Mercury 400 and 600 instruments, operating at 400 or 600 MHz for ^1H NMR and 100 or 150 MHz for ^{13}C NMR. Coupling constants (*J* values) are reported in hertz. Electrospray mass spectra (ESI-MS) were acquired on Agilent 1100 Series LC/MS ion trap mass spectrometers and 6530 Accurate-Mass QTOF spectrometer coupled to an Agilent

HPLC 1200 series (Agilent Technologies). UV-vis spectra were recorded on an Analytik Jena Specord 210 spectrophotometer. Kinetics of real-time assay of pyrophosphatase were recorded on an Agilent Cary 100 UV-vis spectrophotometer.

Synthesis of 4. To a 100 mL round-bottom flask was added 4-hydroxyacetanilide (**3**, 302 mg, 2.0 mmol), ethanol (50 mL), paraformaldehyde (150 mg, 5.0 mmol) and bis-(2-pyridylmethyl)-amine (877 mg, 4.4 mmol). After the reaction mixture was refluxed for over 30 h, the solvent was evaporated under reduced pressure. To the residue was added dichloromethane (25 mL), and the organic phase was washed with water (5 mL × 3), and then dried in anhydrous Na_2SO_4. The crude product was purified by silica gel column (eluent: dichloromethane–methanol = 80 : 1 (v/v)) to afford **4** (631 mg, 55% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.14 (3H, s, CH_3), 3.78 (4H, s, $2CH_2$), 3.86 (8H, s, $4CH_2$), 7.12 (1H, m, PyH), 7.41 (2H, s, ArH), 7.48 (4H, d, J = 5.2 Hz), 7.56 (1H, br, NH), 7.60 (4H, m, PyH), 8.51 (4H, d, J = 2.8 Hz, PyH). ^{13}C NMR (150 MHz, $CDCl_3$): δ 24.2, 54.4, 59.4, 121.4, 122.0, 123.1, 123.8, 129.6, 136.6, 148.7, 152.4, 158.7, 168.3. IR (KBr, cm^{-1}): 3256, 3065, 2924, 1677, 1592, 1569, 1478, 1433, 1373, 1151, 762. MS (ESI): 574.2 (M + H^+), 596.1 (M + Na^+). HRMS (ESI): m/z calcd for $C_{34}H_{36}N_7O_2^+$ $[M + H]^+$ 574.2925, found 574.2964.

Synthesis of 5. A solution of **4** (574 mg, 1.0 m mol) in 10 mL of 6.3 M aq. HCl was heated to reflux for 3 h. After cooling, the solution was basified with 2 M aq. NaOH solution to pH = 9. The neutralized product was extracted into dichloromethane (10 mL × 3), dried with anhydrous Na_2SO_4, and the solvent was evaporated under reduced pressure. The crude product was purified by silica gel column (eluent: dichloromethane–methanol = 100 : 1 (v/v)) to give a dark brown oil (186 mg, 35% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.74 (4H, s, $2CH_2$), 3.86 (8H, s, $4CH_2$), 6.64 (2H, s, ArH), 7.13 (4H, m, PyH), 7.50 (4H, m, PyH), 7.60 (4H, m, PyH), 8.53 (4H, m, PyH), 10.38 (2H, br, NH_2). ^{13}C NMR (100 MHz, $CDCl_3$): δ 54.8, 59.6, 116.8, 122.0, 123.1, 124.3, 136.6, 137.6, 148.8, 158.7. IR (KBr, cm^{-1}): 3381 (br), 2923, 2823, 1591, 1477, 1434, 1367, 1260, 1151, 1000, 860, 766. MS (ESI): 532.8 (M + H^+), 554.8 (M + Na^+). UV-vis spectra: 50 μM in CH_3CN, Abs = 0.594 (256 nm), 0.161 (302 nm), 0.039 (395 nm, λ_{max}). This compound was used immediately in the next reaction.

Synthesis of 1. 4-Chloro-7-nitro-2,1,3-benzoxadiazole (NBD-Cl, 50 mg, 0.25 mmol) in 5 mL of methanol was added dropwise to a solution of compound **5** (133 mg, 0.25 mmol) and $NaHCO_3$ (21 mg, 0.25 mmol) in 5 mL methanol. After stirring at room temperature for 4 h, the mixture was poured into 40 mL water and extracted with ethyl acetate (10 mL × 5). The organic phase was washed with brine (10 mL), dried over anhydrous Na_2SO_4, and then evaporated under reduced pressure. The crude solid product was recrystallised from dichloromethane/petroleum ether to give the desired product **1** (136 mg, 78%). ^{1}H NMR (600 MHz, $CDCl_3$): δ 3.87 (4H, s, $2CH_2$), 3.92 (8H, s, $4CH_2$), 6.51 (1H, d, J = 8.4 Hz, CH), 7.17 (4H, t, J = 5.4 Hz, PyH), 7.33 (2H, s, ArH)), 7.46 (4H, d, J = 7.8 Hz, PyH), 7.62 (4H, t, J = 7.2 Hz, PyH), 8.24 (1H, d, J = 9.0 Hz), 8.43 (1H, br, NH), 8.55 (4H, d, J = 3.6 Hz, PyH). ^{13}C NMR (150 MHz, $CDCl_3$): δ 54.2, 59.5, 100.3, 122.2, 122.9, 123.8, 124.5, 125.5, 127.2, 136.8, 142.1, 144.1, 144.7, 148.9, 148.8, 155.1, 158.5. IR (KBr, cm^{-1}): 3439, 1592, 1569, 1473, 1435, 1299, 1157, 1075, 996, 950, 861, 546. MS (ESI): 695.3 (M + H^+), 717.1 (M + Na^+). HRMS (ESI): m/z calcd for $C_{38}H_{35}N_{10}O_4^+$ $[M + H]^+$ 695.2837, found 695.2868.

Synthesis of 1·2Zn. To a solution of **1** (26 mg, 0.037 mmol) in 5 mL of MeOH, was added $Zn(NO_3)_2{\cdot}6H_2O$ (22.5 mg, 0.076 mmol), and the mixture was stirred for 30 min at rt. After concentrating under reduced pressure, the obtained solid was recrystallised from MeOH to give sensor **1·2Zn**. Mp: >300 °C. ^{1}H NMR (600 MHz, DMSO-d_6): δ 3.67 (4H, br, $2CH_2$), 4.24 (8H, br, $4CH_2$), 5.86 (1H, s, CH), 6.79 (2H, s, 2CH), 7.56 (8H, br, PyH), 8.01 (4H, br, PyH), 8.66 (1H, d, CH), 8.80 (4H, br, PyH), 10.72 (1H, s, NH). IR (KBr, cm^{-1}): 3443, 3133, 1608 (s), 1659, 1471, 1442, 1384 (s), 1297 (s), 1260, 1124, 1507, 997, 957, 769, 564, 519. UV-vis spectra: 35 μM in 50 mM HEPES buffer solution, pH = 7.4, Abs = 0.344 (370 nm), 0.795 (501 nm, λ_{max}). MS (ESI): 1034.45 (M − NO_3^- + OH^- + Na^+)$^+$. Elemental analysis calcd for $C_{38}H_{34}N_{10}O_4Zn_2{\cdot}(NO_3)_4{\cdot}5H_2O$: C 39.22, H 3.81, N 16.85; found: C 39.21, H 3.47, N 16.63.

Synthesis of 6. The compound **6** (400 mg, 35%) was synthesized from 4-hydroxyacetanilide **3** (302 mg, 2.0 mmol) in ethanol with paraformaldehyde (150 mg, 5.0 mmol) and bis-(2-pyridylmethyl)amine (399 mg, 2.0 mmol) by the same method described for the preparation of compound **4**. ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.13 (3H, s, CH_3), 3.76 (2H, s, CH_2), 3.87 (4H, s, $2CH_2$), 6.85 (1H, d, J = 6.0 Hz, ArH), 7.15–7.17 (4H, m), 7.32–7.34 (3H, m), 7.63 (2H, t, J = 4.4 Hz, PyH), 8.56 (2H, t, J = 2.8 Hz, PyH). ^{13}C NMR (150 MHz, $CDCl_3$): δ 24.1, 56.6, 58.7, 116.5, 121.4, 122.2, 122.4, 122.7, 122.8, 123.2, 136.8, 148.7, 154.1, 157.9, 168.5. IR (KBr, cm^{-1}): 3417, 3289, 2961, 1652, 1592, 1566, 1499, 1434, 1371, 1256, 1154, 1084, 756. MS (ESI): 363.1 (M + H^+), 385.0 (M + Na^+). HRMS (ESI): m/z calcd for $C_{21}H_{23}N_4O_2^+$ $[M + H]^+$ 363.1816, found 363.1826.

Synthesis of 7. The compound **7** (132 mg, 78%) was synthesized from **6** (190 mg, 0.52 mmol) by the same method described for the preparation of compound **5**. ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.70 (2H, s, CH_2), 3.85 (4H, s, $2CH_2$), 6.47 (1H, d, J = 2.4 Hz), 6.56 (1H, dd, J = 2.8, 2.8 Hz, ArH), 6.75 (1H, dd, J = 2.0, 2.0 Hz, ArH), 7.15 (2H, t, J = 3.2 Hz, PyH), 7.35 (2H, m, PyH), 7.59–7.64 (2H, m, PyH), 8.56 (2H, d, J = 2.8 Hz, PyH). ^{13}C NMR (150 MHz, $CDCl_3$): δ 57.0, 59.1, 116.3, 116.9, 117.5, 122.2, 123.2, 136.7, 138.1, 148.8, 150.2, 158.2. IR (KBr, cm^{-1}): 3209, 2923, 2826, 1591, 1498, 1433, 1372, 1255, 1152, 764. MS (ESI): 321.1 (M + H^+), 343.0 (M + Na^+). HRMS (ESI): m/z calcd for $C_{19}H_{21}N_4O^+$ $[M + H]^+$ 321.1710, found 321.1727.

Synthesis of 2. By the same procedure described for the synthesis of **1**, the product **2** (190 mg, 98% yield) was synthesized from **7** (132 mg, 0.41 mmol) with NBD-Cl (82 mg, 0.41 mmol). ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.74 (2H, s, CH_2), 3.84 (4H, s, $2CH_2$), 6.53 (1H, d, J = 8.8 Hz), 6.95 (1H, d, J = 8.8 Hz), 7.20 (1H, d, J = 2.4 Hz), 7.23 (1H, d, J = 2.4 Hz), 7.28 (2H, t, J = 6.0 Hz, PyH), 7.41 (1H, d, J = 2.4 Hz), 7.48 (2H, d, J = 7.2 Hz, PyH), 7.76 (2H, t, J = 8.4 Hz, PyH), 8.38 (1H, t, J = 8.8 Hz), 8.52 (2H, d, J = 5.2 Hz, PyH), 10.87 (1H, br, NH).

^{13}C NMR (150 MHz, DMSO-d_6): δ 53.7, 58.6, 101.2, 116.6, 121.6, 122.3, 122.7, 124.5, 124.8, 125.9, 128.6, 136.8, 137.4, 143.3, 144.2, 144.7, 148.7, 156.0, 158.3. IR (KBr, cm^{-1}): 3442, 1570, 1497, 1430, 1299, 1159, 1070, 861, 547. MS (ESI): 484.2 (M + H$^+$), 506.0 (M + Na$^+$). HRMS (ESI): *m*/*z* calcd for $C_{25}H_{22}N_7O_4^+$ [M + H]$^+$ 484.1728, found 484.1738.

Synthesis of 2·Zn. **2·Zn** was prepared in 71% yield using the same method described for the preparation of **1·2Zn**. Mp: 232–235 °C. ^{1}H NMR (600 MHz, DMSO-d_6): δ 3.68 (2H, s, CH_2), 4.08–4.24 (4H, br, $2CH_2$), 6.47 (1H, d, CH), 6.84 (1H, s, CH), 7.17 (2H, s, 2CH), 7.55 (2H, s, PyH), 7.64 (2H, s, PyH), 8.07 (2H, s, PyH), 8.59 (1H, s, CH), 8.91 (2H, s, PyH), 10.48 (1H, br, s), 10.93 (1H, s, NH). IR (KBr, cm^{-1}): 3441, 3133, 1608, 1659 (s), 1442, 1304, 1262, 1122 (s), 1071 (s), 995, 957, 865, 617, 539, 518. UV-vis spectra: 50 μM in 50 mM HEPES buffer solution, pH = 7.4, Abs = 0.147 (366 nm), 0.371 (503 nm, λ_{max}). MS (ESI): 546.6 (M − NO_3^-)$^+$, 587.6 (M − NO_3^- + OH^- + Na^+)$^+$.

Conclusions

In summary, we have developed a new NBD-phenol based colorimetric sensor, which shows a high selective coloration for PPi in pure aqueous solution over a wide pH range. This sensor shows high affinity ($K_a \approx 3 \times 10^8$ M^{-1}) and selectivity for PPi over other anions, which enables the detection of PPi in the presence of a large excess of ATP and inorganic phosphate Pi, and provides a convenient way of assaying pyrophosphatase in real time. In addition, it's worth noting that this is the first use of the NBD-phenol as the chromophore in a colorimetric sensor. The combination of its convenient synthetic incorporation and its absorption at long wavelength (~500 nm) mean that it should be applicable to a variety of other colorimetric sensors.

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