Naphthalene Carbohydrazone Based Dizinc(II) Chemosensor for a Pyrophosphate Ion and Its DNA Assessment Application in Polymerase Chain Reaction Products

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Ⓢ *Supporting Information*

ABSTRACT: A new naphthalene carbohydrazone based dizinc(II) complex has been synthesized and investigated to act as a highly selective fluorescence and visual sensor for a pyrophosphate ion with a quite low detection limit of 155 ppb; this has also been used to detect the pyrophosphate ion released from polymerase-chain-reaction.

The development of new fluorogenic receptors that are highly selective and sensitive for anions is a promising research area of host−guest chemistry because they are ubiquitous in biological systems and play significant roles in a wide area of biology, clinical diagnostics, and environmental monitoring.[1,2] In particular, sensing the pyrophosphate ion (PPi) is biologically important because it is the product of adenosine triphosphate (ATP) hydrolysis under cellular conditions[3] and it participates in DNA and RNA polymerizations and several bioenergetic and metabolic processes.[4] In light of this, detection of PPi is being investigated as a real-time DNA-sequencing method and an invaluable tool in cancer research.[5] Biological fluids such as blood serum contain ∼0.80−1.45 mM phosphates, and the higher phosphate levels are responsible for cardiovascular disease and acute renal failure.[6] As a consequence, many researchers have considered the detection and discrimination of this biologically potent PPi as important fields of research.[7] Various approaches utilizing hydrogen bonding, anion−π interaction, and the chelation mode of interactions have been adapted to the development of fluorescent chemosensors for pyrophosphate,[7,8] phosphate ions,[9] ATP/guanosine 5'-triphosphate (GTP),[9a,10] or phosphorylated peptide.[11] However, rather few have the simplicity and accessibility that is ideally required for practical devices. Recently, metal-ion complexes have been used as receptors for phosphates and have emerged as one of the most successful strategies.[7,8,12] Among the metal-complex-based receptors, the ones that exhibit fluorescence enhancement in the presence of Zn^{2+} have attracted considerable attention because of the strong affinity of Zn^{2+} toward phosphates.[13] Bearing all of these issues in mind, herein we communicate a novel naphthalene carbohydrazone based fluorescent and colorimetric chemosensor R−Zn^{2+} for PPi with a low detection limit even in the presence of several other anions and its potential application in enzymatic fluorescent detection of DNA in polymerase chain reaction (PCR) products.

The naphthalene carbohydrazone based precursor compound PC−R was synthesized in quantitative yield by Schiff base condensation of 2,6-diformyl-4-methylphenol and 1-hydroxynaphthalene-2-carbohydrazide in ethanol at 85 °C. The Zn^{II} ions containing the fluorescent receptor R−Zn^{2+} (Scheme 1) have

Scheme 1. Synthesis of R−Zn^{2+}
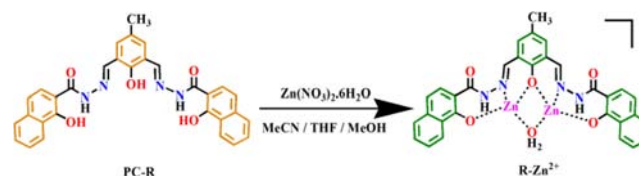
been synthesized by reacting a methanolic solution of Zn-(NO$_3$)$_2$·6H$_2$O with a CH$_3$CN−tetrahydrofuran solution of PC−R at 25 °C. The structures of PC−R and metal-based receptor R−Zn^{2+} were characterized by Fourier transform IR, multinuclear (^{1}H and ^{13}C) NMR, high-resolution mass spectrometry (HRMS), electrospray ionization mass spectrometry (ESI-MS), and elemental analysis (Figures S1−S6, Supporting Information, SI). These spectroscopic and analytical data are consistent with the proposed structural formulas of PC−R and R−Zn^{2+}. The binding behavior of the receptor R−Zn^{2+} toward different anions was monitored using electronic, fluorescence, and ^{31}P NMR spectroscopy. All of the titration studies were carried out in a 0.02 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer at pH = 7.4. The electronic absorption spectrum of R−Zn^{2+} (5 μM) exhibited four sharp bands at 292, 329, 353, and 442 nm (Figure 1).

Upon the gradual addition of an aqueous solution of PPi in increasing concentration (0−50 μM), the intensity of the R−Zn^{2+} peak at 353 nm shows a significant increment in the initial absorption intensity.

The appearance of four well-defined isosbestic points centered at λ = 270, 328, 378, and 427 nm for R−Zn^{2+} is consistent with the existence of an equilibrium between the receptor R−Zn^{2+} and R−Zn^{2+}/PPi in solution. Nonlinear regression analysis of the absorption spectral data (Figure 1A, inset) showed the formation of a 1:1 (PPi/R−Zn^{2+}) stoichiometric complex [log $\beta_{R-Zn^{2+}/PPi}$ = 5.12 ± 0.15]. Notably, the addition of other anions [F$^-$, Cl$^-$, Br$^-$, I$^-$, CH$_3$COO$^-$, HCO$_3^-$, NO$_2^-$, SO$_4^{2-}$, PO$_4^{3-}$, adenosine
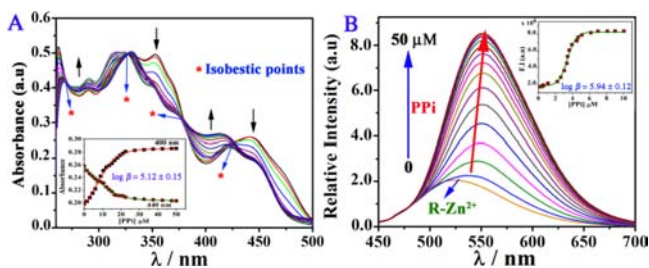
Figure 1. Change in the absorption (left) and fluorescence spectra (right) upon a gradual increase in the concentration of PPi (0−50 μM). Inset: Fitting of (left) absorption (at 400 and 440 nm) and (right) fluorescence changes for the titration of R−Zn^{2+} with PPi.

diphosphate (ADP), and ATP] did not alter the initial absorption spectrum (Figure S7, SI) of the receptor R−Zn^{2+} significantly. From these UV−vis studies, it is clear that the receptor R−Zn^{2+} shows very high selective binding affinity in the ground state only for PPi even in the presence of different other anions.

The fluorescence spectrum of the receptor R−Zn^{2+} (5 μM) exhibits a weak emission at 525 nm in 0.02 M HEPES at pH = 7.4 upon excitation at 440 nm. This fluorescence emission was not changed upon the addition of excess PO$_4^{3-}$, CH$_3$COO$^-$, HCO$_3^-$, etc. In contrast, the PPi anion caused the emission band to be shifted to a longer wavelength at 552 nm, which was significantly enhanced (∼8.2-fold increase) after the addition of ∼1.0 equiv of PPi (Figure 1). This dramatic enhancement of the initial fluorescence intensity of R−Zn^{2+} is due to the selective chelation-enhanced fluorescence (CHEF) effect with PPi. Nonlinear regression analysis of the spectral data obtained upon titration of the solution of R−Zn^{2+} with PPi showed the formation of a 1:1 stoichiometric complex [log $\beta_{R-Zn^{2+}/PPi}$ = 5.94 ± 0.12; Figure 1B, inset]. Stoichiometry plot (Figure 2) analysis of the fluorescence
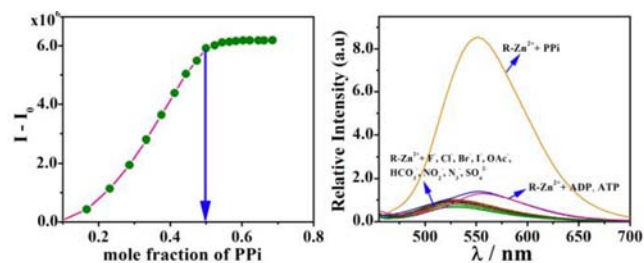

Figure 2. Stoichiometry plot (left). Change in the initial fluorescence intensities of the receptor R−Zn^{2+} (5 μM) in the presence of 1.0 equiv of different anions in 0.02 M HEPES at pH = 7.4 (right).

titration profile of R−Zn^{2+} (5 μM) also revealed a 1:1 stoichiometry between R−Zn^{2+} and PPi species. The formation of a 1:1 complex was further supported by ESI-MS data (Figure S6, SI), which detected the complex at m/z 278.27 corresponding to [C$_{31}$H$_{21}$N$_4$O$_{12}$P$_2$Zn$_2$]$^{3-}$ (=[R−Zn^{2+} + PPi − 3K$^+$]$^{3-}$). To further prove the chelation mode of complexation, a ^{31}P NMR study was performed. As shown in Figure S8 (SI), both P atoms in PPi are magnetically equivalent and show a single signal at −6.37 ppm. However, upon binding with R−Zn^{2+}, a significant downfield shift ($\Delta\delta$ = 3.13 ppm) was observed for the ^{31}P signal, which indicates that R−Zn^{2+} directly interacts with the phosphate sites.[7d,e]

In order to prove the selectivity of the receptor R−Zn^{2+} toward PPi, we carried out fluorescence titration experiments of R−Zn^{2+} with other anions (F$^-$, Cl$^-$, Br$^-$, I$^-$, CH$_3$COO$^-$, HCO$_3^-$, NO$_2^-$,

SO$_4^{2-}$, PO$_4^{3-}$, ADP, and ATP). As shown in Figure 2, only PPi elicited significant fluorescence-enhancing responses, while the other tested anions exhibited almost no fluorescence response under spectroscopic conditions identical with those used for PPi. Thus, receptor R−Zn^{2+} could be used as a highly selective fluorescence sensor for PPi over other anionic species in an aqueous medium. To verify the applicability of the receptor R−Zn^{2+} as selective fluorescence probes for PPi, we carried out a competitive fluorescence titration study with other competing anions.

As shown in Figure 3, the initial fluorescence intensity of R−Zn^{2+} did not change significantly (red bars) upon mixing of the
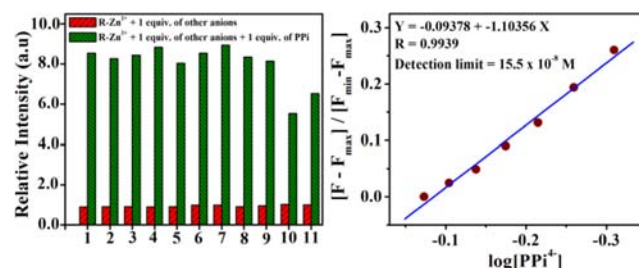

Figure 3. Competitive selective binding affinity of the receptor R−Zn^{2+} (5 μM) toward PPi in the presence of 1.0 equiv of different anions in 0.02 M HEPES (pH = 7.4): from 1 to 11: F$^-$, Cl$^-$, Br$^-$, I$^-$, CH$_3$COO$^-$, HCO$_3^-$, NO$_2^-$, SO$_4^{2-}$, PO$_4^{3-}$, ADP, and ATP) (left); detection limit plot (right).

receptor R−Zn^{2+} with 1 equiv of different other aforesaid anions. However, the subsequent addition of 1 equiv of a PPi solution elicited a prominent fluorescence enhancement (green bars), which further confirmed the excellent selectivity of sensor R−Zn^{2+} for PPi in an aqueous medium even in the presence of other aforesaid interfering anions. Although related phosphate analogues such as ADP and ATP slightly increased the fluorescence intensity, their binding affinity toward R−Zn^{2+} was not sufficiently strong to enable CHEF. Moreover, the search for visual sensors for the trace detection of desired analytes has been a popular target in modern chemistry because of their ease of interpretation and a more suitable tool to practice in the field. The ability of the receptor R−Zn^{2+} as a colorimetric probe for PPi was imaged using a hand-held camera in the presence of other competing anions. As depicted in Figure S9 (SI), R−Zn^{2+} exhibited a distinct visual color change from colorless to pale yellow (under room light) and green emission to almost dark yellow (under UV light) after the addition of a PPi solution. However, there was no observable color change noticed upon mixing of R−Zn^{2+} with other aforesaid interfering anion(s)/solution(s). Thus, the receptor R−Zn^{2+} would probably be suitable as a selective colorimetric sensor for PPi over other competing anions in various environmental and biological systems. In addition, the fluorescence titration profiles also demonstrate that the receptor R−Zn^{2+} has a detection limit of 155 × 10^{-9} M (155 ppb) for PPi, which is lower than that of many reported PPi chemosensors.[9]

Pyrosequencing is a recently developed novel and revolutionary DNA assessment technique[14] that is a simple and quick, one-step, homogeneous phase detection method used to assess DNA amplification after PCRs. This method relies on the enzymatic fluorescent detection of the PPi released from dNTPs, which occurs stoichiometrically when DNA is synthesized by the action of DNA polymerase. The application requires the

fluorescent probe to specifically recognize the released PPi in the presence of structurally similar anionic nucleotides A (Figure 4a).
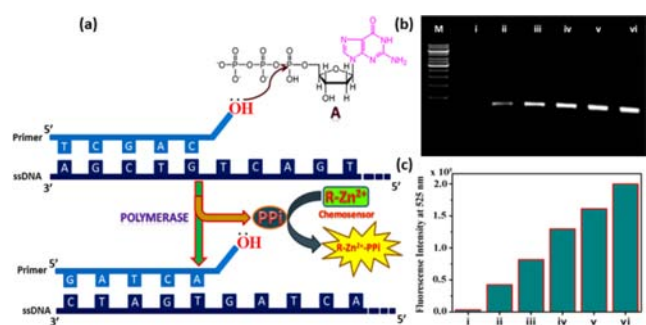

Figure 4. (a) Mechanism of sensing PPi released from PCR by R−Zn^{2+}. (b) Gel electrophoresis of finished PCR mixtures. M = DNA marker. (c) Fluorescence intensity of the sensor R−Zn^{2+} at 525 nm upon addition of the finished PCR product mixture. (i) 10 μL of the finished PCR product mixture performed without template DNA; 10 μL of the finished PCR product mixture performed with template DNA after (ii) 29 cycles, (iii) 30 cycles, (iv) 31 cycles, (v) 32 cycles, and (vi) 35 cycles.

Here, we have used R−Zn^{2+} as the PPi-detecting fluorescent probe because R−Zn^{2+} can detect a small amount of PPi in the presence of a large excess of ATP. Gel electrophoresis revealed the production of DNA of same molecular weight (Figure 4b), where the band intensity was proportional to the amount of PPi released from PCR, and subsequently detected by the fluorescence intensity at 525 nm (Figure 4c). This confirms the hypothesis that the extent of the fluorescence change in R−Zn^{2+} is proportional to not only the amount of PPi generated from PCR but also the DNA amplified. This method might be a simple and swift alternative for assessing the product of PCR, and also the sensor R−Zn^{2+} has potential application in the new generation of DNA sequencings by feature of its selectivity and sensitivity.

In conclusion, we have designed and developed a simple but efficient naphthalene carbohydrazone based colorimetric and fluorescence sensor R−Zn^{2+}, which shows a highly selective and sensitive fluorescence response toward PPi over structurally similar phosphates ADP and ATP and other anions in a 100% aqueous medium. The excellent detection limit (155 ppb) of this fluorescent chemosensor R−Zn^{2+} would be useful in the detection of a trace quantity of PPi in biological and environmental samples. Excellent fluorescence response in addition to its selectivity toward PPi makes R−Zn^{2+} a potential sensor of fluorescent detection and assessment of DNA in PCR products.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental procedures, IR, NMR, HRMS, and ESI-MS spectral data of the compounds, and sensor studies. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

This manuscript was written through contributions of all authors and approved to the final version of the manuscript. These authors contributed equally.

Notes

The authors declare no competing financial interest.

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