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Highly selective visual distinction of pyrophosphate from other phosphate anions with 4-[(5-chloro-2-pyridyl)azo]-1,3-diaminobenzene in the presence of copper(II) ions

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ABSTRACT

Pyrophosphate (PPi), as a biologically related phosphate anion, plays very important roles in organisms. Here, a highly selective visual method for distinction of PPi was made with commercial available 4-[(5-chloro-2-pyridyl)azo]-1,3-diaminobenzene (5-Cl-PADAB) in the presence of copper(II). The yellow solution of 5-Cl-PADAB exhibits strong absorption at 450.5 nm, and addition of Cu(II) results in a red solution with a new absorption band at 506.0 nm. Upon titration with PPi, the absorption band at 506.0 nm decreases with blueshift, while another new absorption band in the region from 562.0 nm to 750.0 nm appears which gradually splits into two peaks, and the color accordingly changes from red to cyan. Further addition of PPi, the new absorption peaks gradually disappear, and the mixture shows the absorption of 5-Cl-PADAB and recovers to yellow from cyan. This process is highly selective for PPi since other phosphate anions such as nucleotides cannot induce such spectral and color changes. With this method, the detection of PPi concentration in human urine was made with satisfactory results.

1. Introduction

Detection of anions is very important, but it is not so easy compared to that of metal ions [1]. For example, pyrophosphate (PPi), as a biologically related inorganic anion and an important member of the family of phosphate anions, plays very important roles in organisms such as anabolism and energy transduction since it is the product of adenosine triphosphate (ATP) hydrolysis and participates in the synthesis of guanosine 3′-diphosphate-5′-diphosphate (ppGpp) [2,3]. Also, PPi has a close relationship with health and disease such as normal and pathological calcification [4], the regulation of fibrin function [5], and cancer diagnosis and therapy [6]. So researchers have paid considerable attention to the detection of PPi, but the present reported methods are not very satisfactory as stated below.

Generally, there are three strategies reported for the detection of PPi with organic small molecules (OSMs) on the basis of the absorption and fluorescence emission properties of the OSMs [1]. One is that OSMs attached with thiourea [7,8], pyrrole [9,10] or imidazolium [11] as receptor sites can directly bind PPi through hydrogen bonding or electrostatic interactions, which, however, makes the selectivity of PPi not good over other phosphate-containing anions. The second strategy is to establish an indicator displacement system, wherein the indicator can be displaced from the receptor by PPi [12–14]. This approach needs to choose an appropriate indicator and also an appropriate receptor to adjust the selectivity of the system. The third one, which might be the most popular strategy for the detection of PPi, is based on the metal ion coordination. This strategy is efficient since metal ion can form a metal complex with the fluorescent or colored OSM ligand, and also has a strong binding affinity for PPi [1,15–27]. For example, a series of fluorophore-bis(2-pyridylmethyl)amine (DPA)–Zn(II) [1] and -Cu(II) [15,16] complexes have been used for the selective detection of PPi. This strategy usually has good selectivity and has been widely accepted to design new probes for PPi. It is a pity, however, that it generally suffers from complicated synthesis and separation procedures of the ligands.

Herein, by adopting the third strategy and applying one of the most commonly used commercial pyridylazobenzene reagent, 4-[(5-chloro-2-pyridyl)azo]-1,3-diaminobenzene, 5-Cl-PADAB, we establish a highly selective visual method for detection and distinction of PPi. As a commercial chromogenic reagent, 5-Cl-PADAB has been widely used for the determination of metal ions. Occasional reports showed that its Co(II) complex could be employed for the sensitive detection of nucleic acids and selective

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recognition of poly T oligonucleotides with the signal outputs of light scattering and molecular absorption, respectively [28–30]. Different from previous reports, we explored herein the PPi recognition properties of its Cu(II) complex on the basis of Cu(II) coordination interaction with both 5-Cl-PADAB and PPi. The recognition process is highly selective for PPi over other members of the large family of phosphate anions and can be used to visually detect PPi with the naked eyes.

2. Experimental

2.1. Chemicals and materials

Stock solution of 1.0×10^{-3} mol/L 5-Cl-PADAB was prepared by dissolving crystallized 5-Cl-PADAB (Merck, Germany) in dehydrated alcohol. 5.0×10^{-4} mol/L working solution was obtained by diluting the stock solution with water. The copper dichloride ($CuCl_2$) solution of 5.0×10^{-4} mol/L was prepared by dissolving $CuCl_2$ of the commercial product from Hai Jing Chemical Plant (Tianjin, China) in distilled water.

Sodium pyrophosphate (PPi) was purchased from Beibei Chemical Plant (Chongqing, China). Adenosine 5′-triphosphate disodium salt (ATP), cytidine 5′-triphosphate disodium salt hydrate (CTP), uridine 5′-triphosphate trisodium salt hydrate (UTP), and guanosine 5′-triphosphate disodium salt hydrate (GTP) were purchased from Sigma, Guanosine 3′-diphosphate-5′-diphosphate, (ppGpp) was purchased from Trilink (USA). These anions solutions were all prepared by dissolving the commercial products into doubly distilled water. Solution concentrations of ATP, GTP, CTP, UTP and ppGpp were 3.0×10^{-4} mol/L, but PPi was 4.0×10^{-4} mol/L. In addition, hexamethylenetetramine (HMTA) solution of 5.0×10^{-4} mol/L as the reaction medium was used. All reagents were of analytical grade without further purification. Doubly distilled water (18.2 MΩ) was used throughout.

2.2. Instrumentation

Absorption spectra were measured with UV-3600 spectrophotometer (Tokyo, Japan). QL-901 vortex mixer (Qilinbeier instrument manufacture Ltd., Haimen, China) was used to blend the solutions. And the images of the color changes of solution were recorded with a Nikon Coolpix-4500 digital camera.

2.3. Pretreatment of the urine samples

The three urine samples collected from three healthy volunteers were centrifuged for 20 min at 12,000 r/min and the supernatant solutions were filtered with 0.45 μm membranes, respectively. The obtained filtrates were transferred for further detection.

2.4. Procedures of titration with anions

50.0 μL 5-Cl-PADAB solution and 50.0 μL Cu^{2+} solution were mixed in a 1.5 mL plastic tube. Then an appropriate amount of PPi or other anions or the real sample and 100 μL HTMA was added, respectively. Finally, the mixture was diluted to 500 μL with doubly distilled water and blended thoroughly. Then the mixture was transferred to a quartz cell for measurements. All absorptions were measured by UV-3600 at 643.0 nm with slit width at 5.0 nm.

3. Results and discussion

3.1. Optimal conditions for the reaction

We carried out the reaction in the medium of hexamethylenetetramine (HMTA) at near-neutral pH, not in some buffer since it was observed that precipitation occurred about 10 min after mixing Cu(II)–5-Cl-PADAB with PPi in the buffers such as Tris–HCl, Britton–Robinson, phosphate buffer, or HAc–NaAc, and in the aqueous media such as Tris, NaOH and HCl. Appropriate content of HMTA can provide a good circumstance for the reaction, and has neglectable influence on the absorption spectra (Supporting information, Fig. S1). However, HMTA higher than 1.0×10^{-4} mol/L can result in significant complexation with Cu(II), exerting adverse effects on the interaction of Cu(II)–5-Cl-PADAB and PPi (Supporting information, Fig. S2). Further experiments showed that the molar ratio of Cu(II) and 5-Cl-PADAB should be kept as 1:1 in the mixture (Supporting information, Fig. S3), and the optimal order of adding reagents is as follows: mixing 5-Cl-PADAB with Cu(II) first, and then PPi, HMTA and H_2O can be added in turn (Supporting information, Fig. S4).

3.2. Spectral characteristics

Fig. 1 shows the absorption spectra of 5-Cl-PADAB in the medium of HMTA in the presence of Cu(II) and PPi, respectively. The yellow homogeneous solution of 5-Cl-PADAB exhibits strong absorption at 450.5 nm, and addition of Cu(II) results in a red solution with a new absorption band at 506.0 nm. Upon further titration with PPi, however, the absorption band at 506.0 nm gets decreased gradually, forming another new absorption band in the region from 562.0 nm to 750.0 nm with an isosbestic point at 562.0 nm, indicating the formation of a new species. With continuous increase of PPi, the absorption at 506.0 nm displays a blueshift to near 465.0 nm and the absorption in the region from 562.0 nm to 750.0 nm starts to split into two peaks. During this process, the color displays a dramatic change from red to cyan. On the contrary, both Cu(II) and PPi solutions have no absorption bands in the region of 300.0–750.0 nm (Supporting information, Fig. S1). Furthermore, this spectra and color change of Cu(II)–5-Cl-PADAB is highly specific for PPi even though Co(II) and Ni(II) can induce obvious color and spectral changes of 5-Cl-PADAB, but the

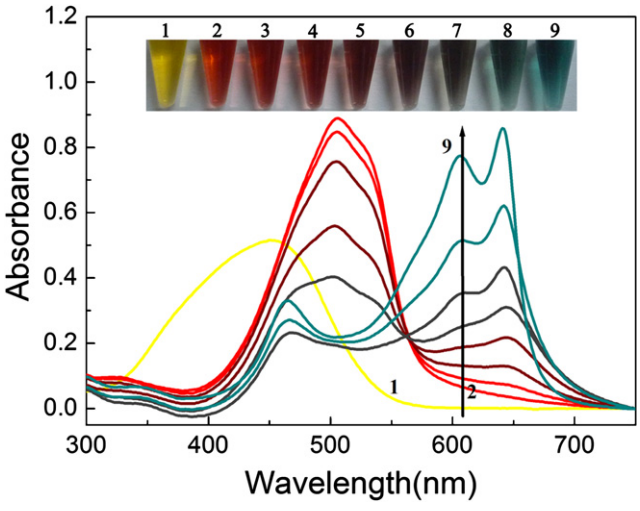


Fig. 1. Absorption spectra of 5-Cl-PADAB (Curve 1) and Cu(II)–5-Cl-PADAB in the presence of PPi (Curves 2–9) in the medium of HMTA. Inset is the corresponding naked color changes. $c_{5\text{-Cl-PADAB}}$, 5.0×10^{-5} mol/L; $c_{Cu(II)}$, 5.0×10^{-5} mol/L; c_{PPi} (from curve 2 to 9), 0, 2.0, 5.0, 10, 15, 20, 25, and 35 μmol/L; c_{HMTA}, 1.0×10^{-4} mol/L.

addition of PPi scarcely induce further spectral changes (Supporting information, Fig. S5).

3.3. Investigations of the interaction mechanism

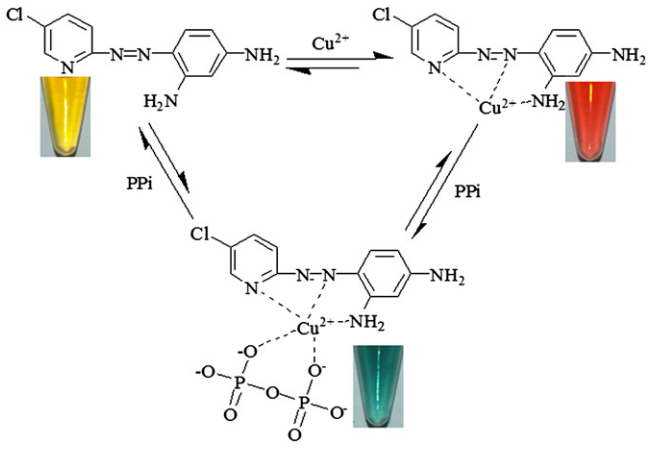
By keeping a fixed concentration of Cu(II) and 5-Cl-PADAB at 5.0×10^{-5} mol/L and changing the concentration of PPi, the new absorption peaks of the Cu(II)–5-Cl-PADAB complex in the region of 562.0 nm–750.0 nm is gradually increased with the concentration of PPi (Fig. 1), which will reach the maximum when the concentration of PPi is up to 5.0×10^{-5} mol/L. Further increasing the amounts of PPi, the new absorption peaks gradually disappear, and the mixture just shows the absorption spectra of 5-Cl-PADAB alone (Fig. 2A) and the colors of the solution recover to yellow from cyan, indicating that the replacement of 5-Cl-PADAB in its Cu(II) complex by PPi has occurred. Fig. 2B shows the Job plot analysis of the titration experiment via addition of PPi to the complex of Cu(II)–5-Cl-PADAB, which reveals the stoichiometry of the Cu(II)–5-Cl-PADAB complex with PPi to be 1:1, and the calculated association constant is $8.5\times10^{3}\ M^{-1}$.

Scheme 1 displays the proposed binding modes of Cu(II)–5-Cl-PADAB complex with PPi. As a pyridylazo benzene dyestuff, 5-Cl-PADAB is usually complexed with metal ion through the pyridyl nitrogen atom, one of the azo nitrogen atoms and the amino group *ortho* to the azo linkage, while the amino group *para* to the azo linkage only exhibits its electron effects and does not participate in the complexation with metal ion [31,32]. When the complex of Cu(II)–5-Cl-PADAB forms following a 1:1 stoichiometry of Cu(II) to 5-Cl-PADAB, the color of the solution changes from yellow to red, corresponding to the appearance of the new absorption peak at 506.0 nm. While the addition of PPi will result in the formation of a ternary complex of Cu(II)–5-Cl-PADAB-PPi with a color of cyan thanks to the strong binding tendency of Cu(II) towards PPi. The decrease and blue shift of the absorption peak at 506.0 nm, and the new absorption bands in wavelength region 562.0 nm–750.0 nm as well as the isosbestic point at 562.0 nm indicate a balance of the Cu(II)–5-Cl-PADAB complex and the Cu(II)–5-Cl-PADAB-PPi ternary complex. However, the balance will be broken if the amounts of PPi increase. So we propose that there exists a competitive effect between 5-Cl-PADAB and PPi. When the molar ratio of PPi to Cu(II)–5-Cl-PADAB is more than 1:1, PPi will gradually pull Cu(II) out of the complex of Cu(II)–5-Cl-PADAB, and consequently make the 5-Cl-PADAB released to the solution, which can explain the recovery of both the yellow color and the absorption spectrum.

It is worthy of noting that the spectral and color changes described above are not ascribed to the pH values despite the fact that the absorption spectra of 5-Cl-PADAB and its metal complex are pH-dependent [33], and the analyte PPi is a little basic. Our experiments show that an equimolar amount of PPi will not induce a spectral shift of 5-Cl-PADAB (Supporting information, Fig. S1, dotted curves 7 and 11), indicating the basicity of PPi is not enough to interfere the pH values of the reaction system, and the new optical signals should be attributed to the PPi acting as an anionic ligand.

3.4. Selectivity of Cu(II)–5-Cl-PADAB complex for PPi

The interactions of Cu(II)–5-Cl-PADAB complex with inorganic anions and various nucleotides were tested in order to evaluate the selectivity. Fig. 3 shows that inorganic anions including CO_3^{2-}, HCO_3^-, NO_3^-, SO_4^{2-}, F^-, Cl^-, Br^-, I^-, CH_3COO^-, SCN^-, PO_4^{3-}, triphosphate and hexametaphosphate result in no characteristic change in the absorption spectra even at the concentration of 10 equiv. of PPi, which may be attributed to the much weaker binding affinity of these anions with Cu(II) in the complex of Cu(II)–5-Cl-PADAB compared with that of PPi. Meanwhile, ATP, GTP, CTP, UTP and ppGpp as analogs of PPi were also investigated in various concentrations as shown in Fig. 4. Although each of these nucleotides contains two or more phosphate units, they induce no obvious absorption bands in the wavelength region 562.0 nm–750.0 nm as well as no obvious color changes (Supporting information, Fig. S6–S10). And therefore, the association constant of these nucleotides with Cu(II)–5-Cl-PADAB complex could not be obtained. According to Hong et al. [34], the good selectivity of the complex of Cu(II)–5-Cl-PADAB for PPi against the



Scheme 1. Proposed binding modes of the Cu(II)–5-Cl-PADAB complex with PPi.

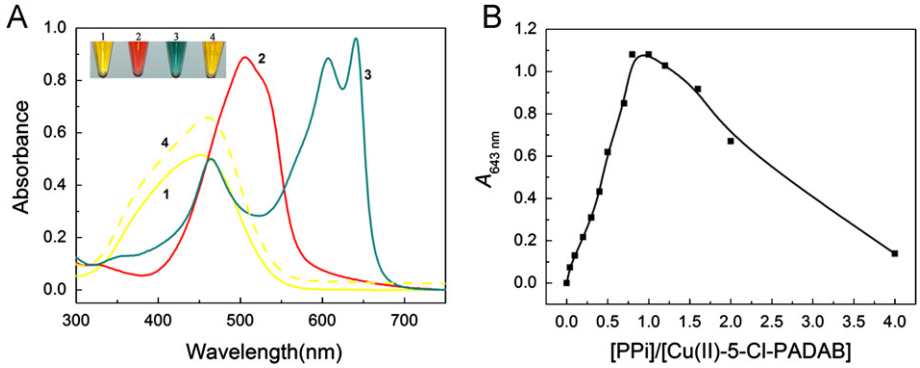


Fig. 2. (A) Absorption spectra of 5-Cl-PADAB (Curve 1) and Cu(II)–5-Cl-PADAB (Curve 2) in the presence of PPi (Curves 3 and 4) in the medium of HMTA. Inset is the corresponding naked color. c_{PPi} (from Curve 3 to 4), 5.0×10^{-5} mol/L, 1.8×10^{-2} mol/L. (B) Job plot analysis of Cu(II)–5-Cl-PADAB complex with PPi. c_{PPi} in turn ($\times10^{-5}$ mol/L), 0, 0.2, 0.5, 1.0, 1.5, 2.0, 2.5, 3.5, 4.0, 5.0, 6.0, 8.0, 10.0, and 20.0. Concentrations: $c_{5\text{-Cl-PADAB}}$, 5.0×10^{-5} mol/L; $c_{Cu(II)}$, 5.0×10^{-5} mol/L; c_{HMTA}, 1.0×10^{-4} mol/L.

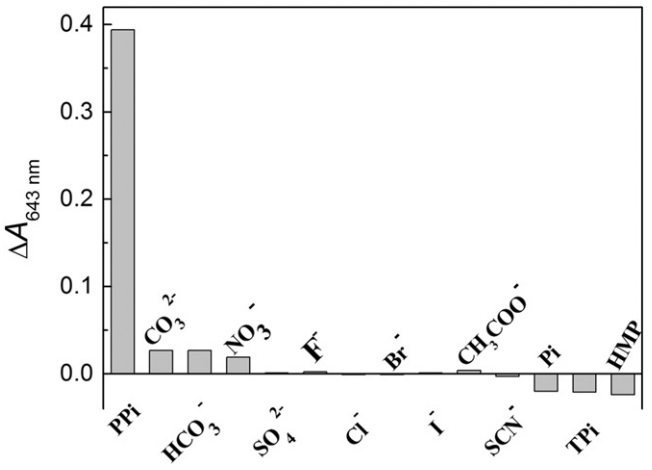


Fig. 3. Comparison of the increase of the absorbance at 643.0 nm induced by adding PPi and CO_3^{2-}, HCO_3^-, NO_3^-, SO_4^{2-}, F^-, Cl^-, Br^-, I^-, CH_3COO^-, SCN^-, PO_4^{3-} (Pi), triphosphate (TPi) as well as hexametaphosphate (HMP) into the complex of Cu(II)–5-Cl-PADAB. c_{PPi}, 2.0×10^{-5} mol/L; c_{others}, 2.0×10^{-4} mol/L. Concentrations: $c_{5\text{-Cl-PADAB}}$, 5.0×10^{-5} mol/L; $c_{Cu(II)}$, 5.0×10^{-5} mol/L; c_{HMTA}, 1.0×10^{-4} mol/L.

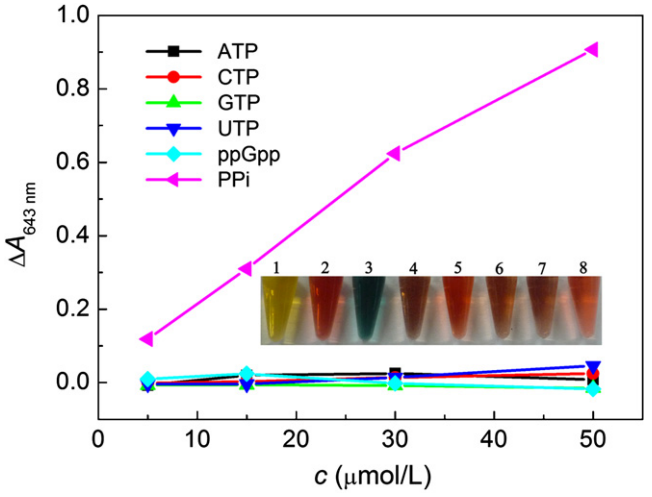


Fig. 4. Concentration-dependent ΔA at 643.0 nm upon addition of PPi, GTP, ATP, CTP, UTP, and ppGpp into the complex of Cu(II)–5-Cl-PADAB. c_{anions} in turn ($\times10^{-5}$ mol/L), 0.5, 1.5, 3.0, and 5.0. Inset is the naked color of 5-Cl-PADAB (1) and Cu(II)–5-Cl-PADAB (2) in the presence of PPi (3), ATP (4), GTP (5), CTP (6), UTP (7) and ppGpp (8) with the same concentration of 5.0×10^{-5} mol/L. Concentrations: $c_{5\text{-Cl-PADAB}}$, 5.0×10^{-5} mol/L; $c_{Cu(II)}$, 5.0×10^{-5} mol/L; c_{HMTA}, 1.0×10^{-4} mol/L.

nucleotides can be ascribed to the smaller charge density of O–P oxygen atoms of these anions involved in complexation with Cu(II) than that of PPi. The results indicate that the complex of Cu(II)–5-Cl-PADAB has unique absorption spectra and color changes upon the addition of PPi, and can act as a useful probe to distinguish PPi from other anions.

3.5. Detection of PPi in human urine

As shown in Fig. 1, the increased absorbance (ΔA) at 643.0 nm of the Cu(II)–5-Cl-PADAB complex was linearly correlated with the concentration of PPi in the range of 2.0–35 μmol/L ($\Delta A = -0.043 + 0.027\ c$ (μmol/L), $r = 0.9935$, from three measurements) with a detection limit of 0.2 μmol/L. In order to test the analytical application of the present method, we collected three urine samples offered by three healthy volunteers. First, the influences of the coexisting substances in urine such as metal ions, amino acids, glucide and urea were investigated within the tolerance level of 10%. The experimental results showed that no remarkable interference in the detection of PPi was induced by these coexisting substances (Supporting information, Table. S1). Further, we applied the Cu(II)–5-Cl-PADAB complex to the detection of PPi concentrations in three urine samples according to the general procedure. The recoveries determined by the external addition of PPi were 90.2–96.6% with the RSD of 0.4–3.9%. (Data were shown in Supporting information, Table. S2).

4. Conclusions

In summary, a highly selective visual method for distinction of PPi from other phosphate anions with 4-[(5-chloro-2-pyridyl) azo]-1,3-diaminobenzene in the presence of copper(II) ions is established. This distinction does not need any complicated modification or organic synthesis and exhibits remarkably good selectivity for PPi over other anions such as phosphate, ATP, GTP, ppGpp and so on. In this approach, Cu(II) is a bridge linking 5-Cl-PADAB and PPi to form a colored complex, which gives a clue to the application of some pyridylazobenzene dyestuffs to the colorimetric determination of anions.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2012.08.046.

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