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A novel NIR fluorescent turn-on sensor for the detection of pyrophosphate anion in complete water system†

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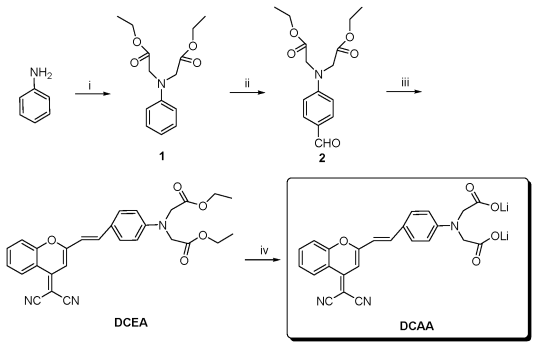
A novel fluorescent sensor DCAA-Cu^{2+} was developed, showing turn-on fluorescence in NIR region with high selectivity to pyrophosphate anion in 100% *aqueous* solution.

The selective recognition and sensing of biologically important anions has attracted great attention for both chemists and biologists.[1,2] Near-infrared (NIR) dyes with emission wavelengths in the region of 650–900 nm are preferable to bioimaging with several advantages, such as penetrating deeper into the tissue with smaller scattering, lower damage of living-cells, and less auto-fluorescence with much lower background signal.[3] As the bioproduct of adenosine triphosphate (ATP) hydrolysis and enzymatic reactions, pyrophosphate anion (PPi) is becoming a biologically important target.[4] Generally, metal complexes can bind anions more efficiently than water, therefore the utilization of a metal ion complex as a binding site for PPi has become the most popular approach.[5] However, the development in fluorescent sensors for PPi under real aqueous physiological conditions is still challenge due to the strong hydration effects and various complexation geometries.[6] To date, there are a few chemical sensors that detect PPi in an aqueous solution,[7] however, the emission spectra are in short wavelength and cannot fall in the NIR region. Accordingly, it is of great significance to develop NIR sensors that can detect PPi selectively and sensitively in a 100% aqueous physiological system.

Bearing this in mind, herein we present a novel colorimetric and fluorescent turn-on PPi sensor **DCAA–Cu^{2+}**, which employs dicyanomethylene-4*H*-chromene as the fluorophore and iminodiacetic acid group as the receptor. Since α-amino acid is a good chelating ligand for Cu^{2+},[8] the incorporation of a lithium iminodiacetate group in **DCAA** (Scheme 1) can play a double role, that is, as the coordinate receptor and to improve water solubility. More importantly, the system can preferably extend the conjugation system with a red shift in emission band to 675 nm, thus making the determining wavelength fall in the desired NIR region. To the best of our knowledge, **DCAA–Cu^{2+}** is the first NIR fluorescent sensor for PPi with the emission wavelengths beyond 650 nm, especially in complete water solution.



Scheme 1 The synthetic route of **DCAA**: (i) $BrCH_2COOEt$, K_2HPO_4/CH_3CN, 75% yield; (ii) $POCl_3$, DMF, 60% yield; (iii) 2-(2-methyl-4*H*-chromen-4-ylidene) malononitrile, 48% yield; (iv) LiOH, MeOH/THF, 48% yield.

As shown in Scheme 1, the sensor precursor **DCEA** was first obtained by a Knoevenagel reaction of 2-(2-methyl-4*H*-chromen-4-ylidene)malononitrile and diethyl 2,2′-((4-formylphenyl)azanediyl)diacetate (**2**), which was synthesized from diethyl 2,2′-(phenylazanediyl)diacetate (**1**) *via* Vilsmeier formylation in 60% yield.[9] Then, the target lithium iminodiacetate **DCAA** was obtained from the hydrolysis of **DCEA** under basic conditions using a modified protocol.[10] The chemical structures were fully characterized by ^{1}H NMR, ^{13}C NMR and HRMS, as shown in the electronic supplementary information (ESI†). The characteristic coupling constant (J = 16.0 Hz) of the protons in ^{1}H NMR of **DCAA** is indicative of the predominate *trans*-isomer (Scheme 1). In electronic spray source HRMS, the high resolution mass peak found at 426.1089 is consistent with the calculated data for $[M - 2Li + H]^-$ (426.1090).

Due to the incorporation of a highly hydrophilic group with the lithium iminodiacetate group, **DCAA** is completely soluble in water. Hence, the fluorescent behavior of **DCAA** toward various metal ions were first checked in a completely aqueous system buffered with 3-(*N*-morpholino) propanesulfonic acid (MOPS, 10 mM, pH = 7.0). As shown in Fig. 1A, there is no obvious change in the fluorescent emission in the presence of K^+, Na^+, Mg^{2+}, Ca^{2+}, Zn^{2+}, Ag^+, Cd^{2+}, Hg^{2+}, Co^{2+}, Mn^{2+}, Ni^{2+} and Sn^{2+}, but only adding Cu^{2+} causes a

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† Electronic supplementary information (ESI) available: Synthesis and full characterization of **DCAA**, Figs S1–S12. See DOI: 10.1039/c2cc16902a

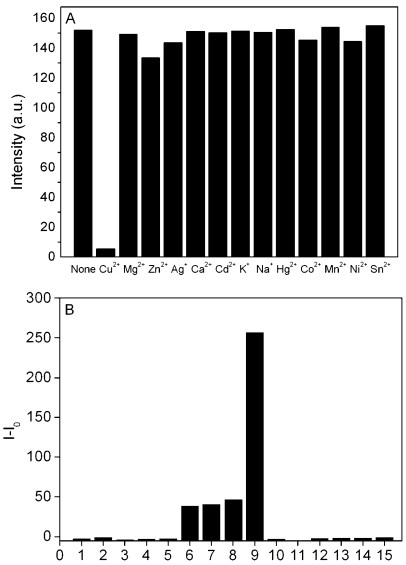

Fig. 1 (A) The fluorescence change of **DCAA** (10 μM) to various metal ions at 50 μM concentration in an aqueous solution buffered with MOPS (10 mM, pH = 7.0) excited at 500 nm. Note: the corresponding perchlorates were utilized as the source of metal cations. (B) The fluorescent response of **DCAA–Cu^{2+}** (10 μM) to various anions at 150 μM concentration in an aqueous solution buffered with MOPS (10 mM, pH = 7.0) excited at the isosbestic point of 450 nm: 1, F^-; 2, Cl^-; 3, Br^-; 4, I^-; 5, CH_3COO^-; 6, $H_2PO_4^-$; 7, HPO_4^{2-}; 8, PO_4^{3-}; 9, $P_2O_7^{4-}$; 10, SO_4^{2-}; 11, NO_3^-; 12, CO_3^{2-}; 13, HSO_3^-; 14, NO_2^-; 15, HCO_3^-.

significant decrease in fluorescence, indicating that **DCAA** can distinguish Cu^{2+} from other metal ions in neutral aqueous buffer solution. Consequently, the copper titration experiment was further investigated. As shown in Fig. S1†, the fluorescent intensity decreased with an increase in Cu^{2+} concentration and complete fluorescence quenching was observed when 5.0 equiv. of Cu^{2+} was added. Moreover, the Job plots with both absorption and fluorescence titrations exhibited a maximum at about 0.33 mol fraction (Fig. S1†), indicating that **DCAA** forms a 2 : 1 complex with Cu^{2+}, which is consistent with the binding mode of corresponding complexes.[11] Also, the mass peak for **DCAA–Cu^{2+}** at *m*/*z* 915.1472 corresponding to $[C_{48}H_{32}N_6O_{10}Cu]^-$ (= [2DCAA + Cu^{2+} + 2H]$^-$ calculated as 915.1476) gives strong evidence that **DCAA** forms a 2 : 1 complex with Cu^{2+} (Fig. S1C†). The binding constant of **DCAA** with Cu^{2+} was determined to be 1.1 × 10^{-6} M^{-1} based on fluorescence titrations.

Interestingly, when adding 1.0 equiv. of **EDTA** solution, the fluorescence intensity was fully restored (Fig. S2†), which can be explained by the difference in association constants between **EDTA–Cu^{2+}** and **DCAA–Cu^{2+}**. As a consequence, the formation of metal complex of **DCAA–Cu^{2+}** is responsible for the fluorescence quenching. The sensitivity to Cu^{2+} was also calculated on the basis of the linear relationship between the emission intensity at 675 nm and the Cu^{2+} concentration, indicating that **DCAA** has a detection limit of 1.26 μM for Cu^{2+} in aqueous solution (Fig. S3†), which is much lower than the typical concentration of blood copper (11.8–23.6 μM) in normal individuals and the limit of copper in drinking water (~20 μM) set by the U. S. Environmental Protection Agency.

Based on the quenching character of Cu^{2+} to **DCAA**, the complex of **DCAA** with Cu^{2+} was prepared *in situ* by mixing **DCAA** with $Cu(ClO_4)_2$ at a 1 : 5 ratio in aqueous solution,[3a] then the selectivity of **DCAA–Cu^{2+}** toward different anions was determined. As shown in Fig. 1B, various anions (1, F^-; 2, Cl^-; 3, Br^-; 4, I^-; 5, CH_3COO^-; 6, $H_2PO_4^-$; 7, HPO_4^{2-}; 8, PO_4^{3-}; 9, $P_2O_7^{4-}$; 10, SO_4^{2-}; 11, NO_3^-; 12, CO_3^{2-}; 13, HSO_3^-; 14, NO_2^-; 15, HCO_3^-) were added into the solution, but only PPi responded obviously to the sensor; other phosphate anions, such as $H_2PO_4^-$, HPO_4^{2-} and PO_4^{3-}, showed a minor disturbance. Apparently, **DCAA–Cu^{2+}** can function as a fluorescent turn-on PPi sensor from other investigated anions, including phosphate anions. However, the system cannot avoid the possible disturbance of peptides and amino acids, such as glycine and histidine at high concentration (Fig. S4†).[12]

PPi titration was then conducted in an aqueous system buffered with MOPS (10 μM, pH = 7.0). When PPi was gradually added to the solution of **DCAA–Cu^{2+}**, a new red-shifted peak at 503 nm appeared and increased gradually with an isosbestic point at 450 nm (Fig. 2A). The associated color change from pale brown to red can be easily distinguished by the naked eye (Fig. 3). Meanwhile, the fluorescence intensity in the NIR region of 675 nm is obviously enhanced (Fig. 2B) and gradually stabilized upon the addition of 15 equiv. of PPi. Choosing the well-known laser dye DCM as a standard (Φ_{DCM} = 0.44),[13] the fluorescence quantum efficiency (Φ) of **DCAA** is calculated to be 0.79 and that of **DCAA–Cu^{2+}** after the addition of PPi is 0.48. Here, the fluorescence can't be recovered to the original intensity of **DCAA**, which indirectly indicates that PPi does not form a strong enough complex to remove Cu^{2+} from **DCAA–Cu^{2+}** (Fig. S5†). It is most likely that one **DCAA** is displaced by PPi, partly restoring the emission of the ligand. Similarly, the detection limit of **DCAA–Cu^{2+}** toward PPi was evaluated to be 2.02 μM using 3 μM **DCAA–Cu^{2+}**, which is superior or comparable to most of the reported PPi sensors (Fig. S6†).[14]

Finally, we applied the NIR sensor of **DCAA–Cu^{2+}** to KB cells (human nasopharyngeal epidermal carcinoma cell) with the use of a confocal fluorescence microscopy to examine the potential application in bioimaging. Bright-field measurements confirmed that the cells after treatment with **DCAA–Cu^{2+}** and PPi were viable throughout the imaging experiments. Incubation of KB cells with 10 μM of **DCAA–Cu^{2+}** for 0.5 h at 37 °C gave nearly no intracellular fluorescence (Fig. 4A–C). After the cells were subsequently supplemented with PPi (30 μM) for 30 min at 37 °C, a remarkable increase of intracellular fluorescence

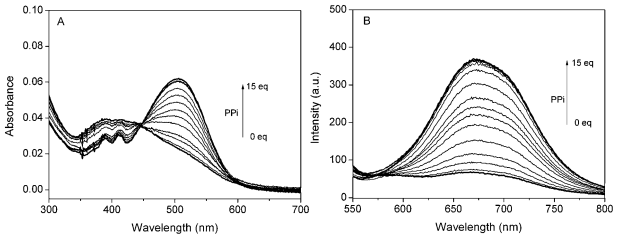

Fig. 2 Spectra of **DCAA–Cu^{2+}** (10 μM) in an aqueous solution buffered with MOPS (10 mM, pH = 7.0) upon titration with 0–15 equiv. PPi: (A) absorption change; (B) emission change excited at the isosbestic point of 450 nm.

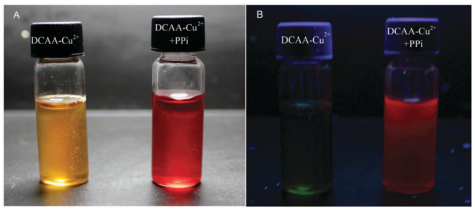

Fig. 3 Photographic images observed from **DCAA–Cu^{2+}** with the addition of PPi ([**DCAA–Cu^{2+}**] = 10^{-4} M, [PPi] = 5×10^{-4} M): (A) color change; (B) fluorescent emission change irradiated at 365 nm by a portable fluorescent lamp.

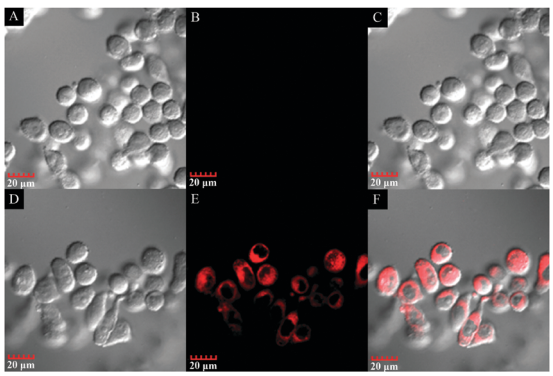

Fig. 4 Confocal fluorescence images in KB cells: Top, (A–C) cells incubated with **DCAA–Cu^{2+}** (10 μM) for 0.5 h. Bottom, (D–F) cells incubated with **DCAA–Cu^{2+}** (10 μM) for 0.5 h, then $K_4P_2O_7$ (30 μM) for 0.5 h. Emission was collected at 630–730 nm upon excitation at 405 nm. Bright field (A and D), fluorescence (B and E) and overlap field (C and F).

was observed (Fig. 4D–F). The overlay of fluorescence and bright-field images reveals that the fluorescence signals are localized in the perinuclear area of the cytosol, indicating a subcellular distribution of PPi anion and good cell membrane permeability of **DCAA–Cu^{2+}**. Moreover, from the bioimaging of cells (Fig. 4A–C), we found that **DCAA–Cu^{2+}** did not show fluorescent enhancement, suggesting that the disturbance of peptide and amino acids in the cell environment can be neglected. This cell experiment demonstrated the potential application of **DCAA–Cu^{2+}** for the imaging of PPi in living cells.

In conclusion, a novel fluorescent sensor **DCAA–Cu^{2+}** was designed on the basis of a dicyanomethylene-4*H*-chromene framework with several merits: (i) detecting PPi anion selectively and sensitively in a pure aqueous physiological system; (ii) being capable of the maximum signal-to-noise ratio with the preferable 'off–on' sensing mode; and (iii) falling NIR determining wavelength to get good transmission and low autofluorescence in biological samples.

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