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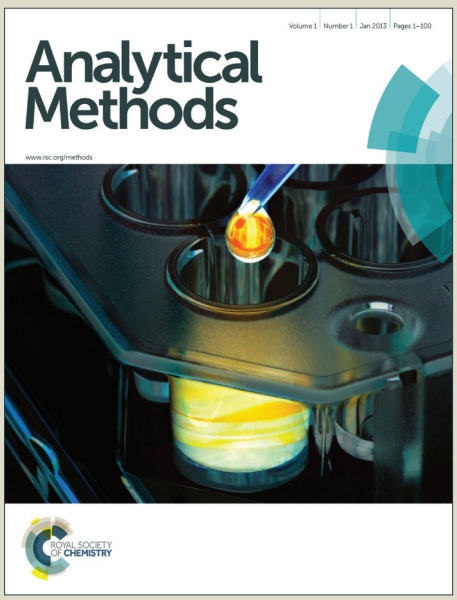

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ARTICLE TYPE

Reactive Probe for Cu^{2+} Based on ESIPT Mechanism and Its Application in Live-Cell Imaging

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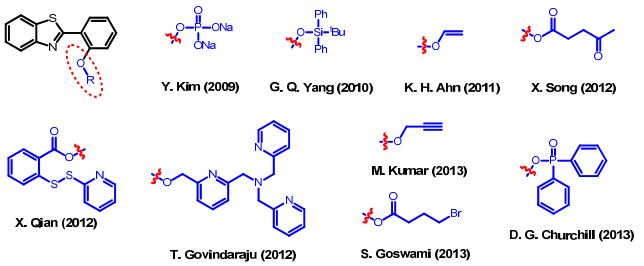
A new reactive probe based on excited-state intramolecular proton transfer (ESIPT) mechanism is reported for the selective detection of Cu^{2+} by the copper induced hydrolysis reaction. The probe is demonstrated to detect Cu^{2+} in living cells.

Copper, which plays crucial roles in many important biological processes such as the neural signal transmitters, gene expression regulators, and several transcription factors, is the third-most abundant transition-metal ion in human body.[1] However, the normal concentration range for copper ions in biological systems is narrow, with both deficiency and excess causing many pathological states, such as myelopathy, neutropenia, Alzheimer's disease, Menkes syndrome, Amyotrophic lateral sclerosis, and Wilson's disease.[2] Therefore, there is a clinical need to quantitatively monitor the existence of Cu^{2+} ions in *vitro* and in *vivo*.

Fluorescent sensor has become an important diagnostic tool for biological and environmental concern for their ease of application in solution as well as their high sensitivity to and selectivity for trace analytes with spatial and temporal resolution.[3] For these purposes, numerous fluorescent probes based-on different receptors and fluorescence units have been developed for Cu^{2+} detection. However, only a few sensors can display "turn-on" or ratiometric manner in emission spectra, most of them show "turn-off" fluorescence response to Cu^{2+} due to the inherent paramagnetic nature of Cu^{2+}.[4]

Compared with the typical-developed chemosensors, fluorescent chemodosimeters, based-on highly specific chemical reaction between the dosimeter and the analyte, have received much attention.[5] More recently, Taki, Govindaraju, and Liu's groups reported reaction-based fluorescence turn-on probes for Cu^{2+} in living cells.[6] However, those probes require laborious synthesis processes and expensive chemicals. Therefore, for practical applications, it is still strongly desirable to develop simple fluorescence turn-on chemodosimeters with excellent performance for Cu^{2+} under physiological conditions.

2-(2′-Hydroxyphenyl) benzothiazole (HBT) is very well-known chromophore exhibiting excited state intramolecular proton transfer (ESIPT) through the keto-enol tautomerism which results in a large Stokes shift. To date, a number of reactive probes have been reported based on the HBT moiety for different analytes *via* "protection-deprotection" strategy



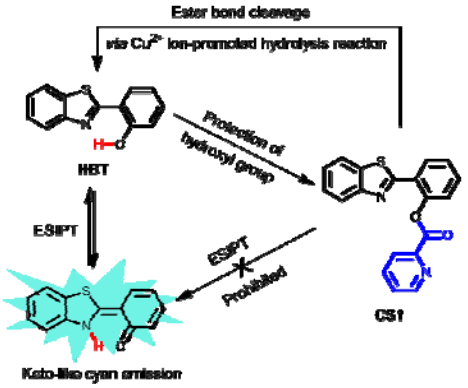
Scheme 1 ESIPT-based HBT probes.

(Scheme 1).[6b,7]

Inspired by these works, we report here a new and simple fluorescence turn-on chemodosimeter **CS1**, picolinoyl-ester derivative of HBT, for the detection of Cu^{2+}. We envisioned that the protection of the hydroxyl group of HBT might prevent the ESIPT process. However, the deprotection of picolinoyl-ester of **CS1** by Cu^{2+} promoted hydrolysis reaction would generate the free HBT moiety which will recover its ESIPT property (Scheme 2).

As shown in Scheme S1 (ESI†), **CS1** can be readily prepared in one convenient step under facile reaction conditions with high yield by coupling of commercially available picolinic acid with HBT. The product (**CS1**) was well characterized by ^{1}H, ^{13}C NMR, and HR-MS (ESI†).

In the UV-vis absorption spectra (Fig. 1), **CS1** exhibits a broad band from 250 to 350 nm. Upon incremental addition of Cu^{2+} (0-1.0 equiv.), the peak at 285 nm slightly decreased, and a new band at 340 nm, which is characteristic of HBT fluorophore, appeared instantly with a clear isosbestic point at



Scheme 2 The "protection-deprotection" strategy for the design of **CS1**.

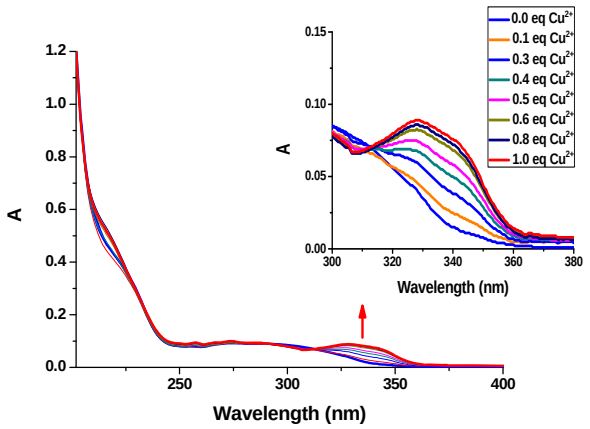


Fig. 1 Absorption spectra of **CS1** (5.0 μM) in PBS buffer solution (10 mM, pH 7.4, containing 20% CH_3CN) in the presence of different concentrations of Cu^{2+} (0-1.0 equiv.).

313 nm. Furthermore, a good linear relationship was observed between the changes in the absorbance at 339 nm with Cu^{2+} in the range of 0-3.0 μM (Fig. S1, ESI†).

The emission spectra of **CS1** and its fluorescence titration with Cu^{2+} were recorded in PBS buffer (10 mM, pH 7.4, containing 20% CH_3CN). As expected, **CS1** alone is almost non-fluorescent (λ_{ex} = 340 nm, Φ = 0.006, Table S1, ESI†). However, upon progressive addition of Cu^{2+}, the emission band at 460 nm rapidly increased, which was attributed to the formation of free ESIPT active HBT fluorophore by Cu^{2+} catalyzed hydrolytic cleavage of the ester bond in **CS1**.[8] Moreover, the fluorescence titration curve revealed that the fluorescence intensity at 460 nm increased linearly with increasing concentration of Cu^{2+} (0-4.5 μM) (R = 0.9905) (Fig. 2 and S2, ESI†) and further smoothly increased until a maximum was reached up to 15.0 μM Cu^{2+} (Φ = 0.028, Table S1, ESI†). Based on these result, the detection limited of **CS1** for Cu^{2+} was determined to be 1.61×10^{-8} M, which was much lower than the limit (20 μM) of Cu^{2+} concentration in drinking water permitted by the U.S. Environmental Protection Agency (Fig. S2, ESI†).

The plausible mechanism of Cu^{2+} induced fluorescence

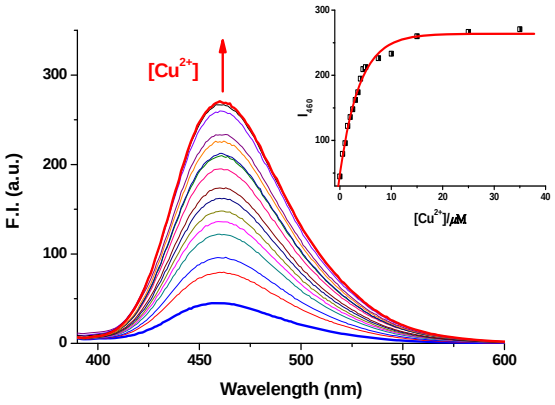


Fig. 2 Fluorescence emission spectra of **CS1** (5.0 μM) in PBS buffer solution (10 mM, pH 7.4, containing 20% CH_3CN) upon the addition of Cu^{2+} (0-15.0 μM) (λ_{ex} = 340 nm). Inset: the fluorescent intensity at 460 nm as a function of Cu^{2+} concentration.

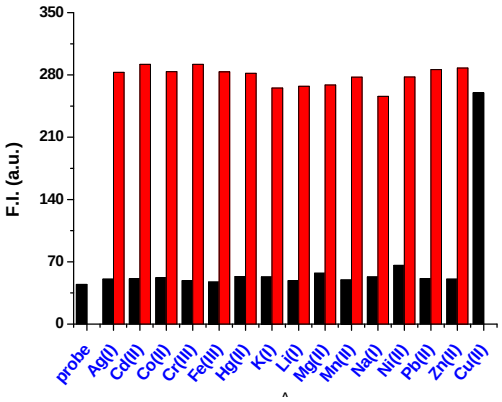


Fig. 3 Fluorescence responses of **CS1** (5.0 μM) with various metal cations in PBS buffer solution (10 mM, pH 7.4, containing 20% CH_3CN). Bars represent the fluorescence intensity of **CS1** in the presence and the absence of various metal cations. Black bars represent the addition of 15.0 μM different metal ions to the solution of **CS1** (5.0 μM). Red bars represent the subsequent addition of 15.0 μM Cu^{2+} to the solution (λ_{ex} = 340 nm).

response is shown in Scheme S2, ESI†. The pyridine N and carbonyl O of picolinic ester in **CS1** provide two coordination sites for Cu^{2+} ions in a way that would strongly activate the carbonyl carbon, which then undergo a nucleophilic addition-elimination reaction with water to release the free HBT moiety. And the HBT is responsible for the cyan emission (λ_{em} = 460 nm, Stokes shift = 118 nm) by ESIPT upon excitation. Efforts were then made to ensure the Cu^{2+} induced hydrolytic cleavage of **CS1** to HBT. We have compared the fluorescence spectra of HBT and the **CS1**-Cu^{2+} system to confirm the generation of HBT after Cu^{2+} treatment (Fig. S7, ESI†). We also carried out the HPLC-MS measurements for the **CS1**-Cu^{2+} solution (Fig. S8, ESI†). All those results agree well with the proposed Cu^{2+} induced deprotection of **CS1**.

Subsequently, the time-dependence of **CS1** fluorescence was also evaluated in the presence of Cu^{2+}. The result shows that the fluorescence of the tested solutions remarkably increased to the maximum value within the 60 minutes. No changes in fluorescence were detected in the absence of Cu^{2+} (Fig. S5, ESI†).

Further, the fluorescence titration of **CS1** with various metal ions was conducted to examine the selectivity (Fig. 3, and S3, ESI†). Much to our delight, the examined alkali, alkaline-earth metal ions, and transition metal ions showed nominal changes in the fluorescence of spectra of **CS1**. It should be mentioned that **CS1** still responds to Cu^{2+} sensitively even in the presence of other relevant competing ions (Fig. 3, and S4, ESI†). Therefore, these results suggest that **CS1** displays high selectivity toward Cu^{2+} in neutral aqueous solution.

pH effects on the fluorescence of **CS1** and the **CS1**-Cu^{2+} system were also investigated. As depicted in Fig. S6, ESI†, **CS1** alone is inert to pH in the range of 4.0-9.0. But in the presence of Cu^{2+}, the fluorescence response of **CS1** decreased when pH of test solutions was lowered, which was due to the protonated nitrogen of picolinic ester in **CS1** lost its ability to bind to Cu^{2+}. On the other hand, it reacted more readily with

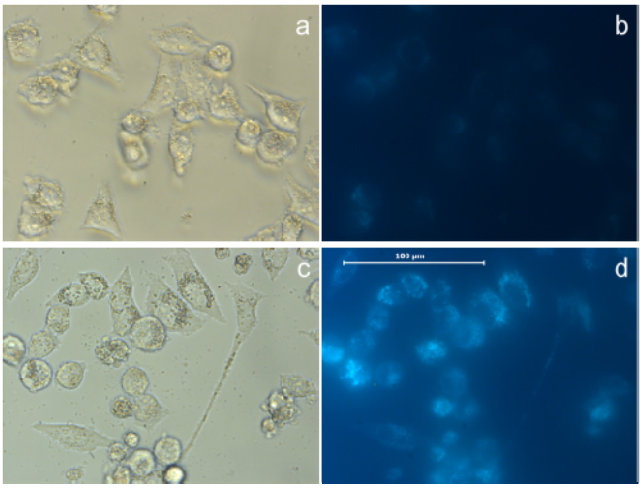


Fig. 4 Fluorescence image of HeLa cells incubated with **CS1** (5.0 μM) for 1 h, and then washed quickly with PBS for imaging (b). The cells were then treated with $CuCl_2$ (15.0 μM) for 1 h which resulted in a dramatic increase in intracellular fluorescence intensity (d). (a), (c) Bright-field images of live cells in (b) and (d).

Cu^{2+} when increased the pH of test solutions, which was attributed to the hydroxide ion (better nucleophile than water) involved into the hydrolysis reaction. These results are also consistent with the proposed mechanism (Scheme S2, ESI†). Moreover, satisfactory Cu^{2+}-sensing abilities were exhibited in the range of pH from 6.0 to 8.0, indicating that **CS1** could be used in living cells without interference from pH effects.

Duo to the favorable properties of **CS1** in vitro, the potential utility of **CS1** in living cells was studied. HeLa cells were incubated with 5.0 μM of **CS1** for 1 h at 37 ^{o}C exhibited weak fluorescence (Fig. 3b). The cells were then treated with $CuCl_2$ (15.0 μM) for 1h at 37 ^{o}C and resulted in a dramatic increase of intracellular fluorescence (Fig. 3d), which indicated that **CS1** was cell membrane permeable and capable of giving image of Cu^{2+} in living cells.

In conclusion, we have rationally developed a new and simple ESIPT-based sensitive fluorescence probe for Cu^{2+} *via* copper triggered hydrolysis reaction. The probe displayed specific fluorescence response towards Cu^{2+} in mild conditions with a low detection limit. Furthermore, fluorescence imaging of Cu^{2+} in living cells indicated that this probe might be favorable for biological applications.

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† Electronic Supplementary Information (ESI) available: Experimental details, characterization of the compounds, and additional spectroscopic data. See DOI: 10.1039/b000000x/

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