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View Article Online
DOI: 10.1039/C5NJ03649F

Journal Name

ARTICLE

A "turn-on" fluorescent probe for detection of Cu^{2+} in living cells based on signaling mechanism of N=N isomerization

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Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

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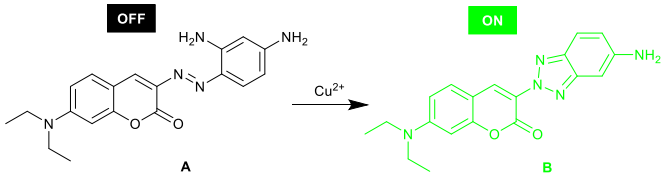
A novel fluorescent probe **A** based on coumarin associated with a N=N double bond was conveniently designed and synthesized for Cu^{2+}, and both rapid colorimetric and fluorescence turn-on detection can be achieved, showing a visible color change from pink to yellow and a significant fluorescence enhancement, respectively. The probe exhibits high selectivity toward Cu^{2+} over other mental ions and excellent sensitivity with a detection limit of 20 nM, and it can be monitored at a wide range of pH (3 - 10) in aqueous system. Besides, the probe has been applied in living cells.

Introduction

Selective detection of metal ions has attracted considerable attention in recent years.[1] Among the various approaches to detecting ions, fluorescent probes are regarded as the most powerful tools due to their selective, sensitive, non-destructive, and real-time monitoring qualities.[2-4] Actually, in the past few years, great efforts have been devoted to the development of fluorescent probes for Cu^{2+} due to its indispensable roles in environmental and biological processes ranging from bacteria to mammals.[5-7] As a transition metal, copper is the third most abundant essential trace element in the body, and usually presents at a low level in cells and tissues.[8] If excessively accumulated to the extent that the cellular homeostasis is broken, copper can cause many seriously adverse neurodegenerative damages, such as Alzheimer's disease and Wilson's disease.[9-11] Owing to its paramagnetic nature, Cu^{2+} is known as an efficient fluorescence quencher,[12-14] and early-reported Cu^{2+} probes generally underwent fluorescence quenching upon binding of Cu^{2+}.[15-19] Fluorescence turn-off probes may exhibit false positive results, while fluorescence turn-on probes possess more excellent advantages.[20] In fact, recent years have witnessed a growing number of fluorescence enhancement probes for Cu^{2+}.[21-24] Unfortunately, in most cases, the fluorescence increase is fairly weak while the background signal is strong. Besides, the poor water solubility often limits the application of the probes in living tissues. Moreover, the low sensitivity and high interference by other closely related metal ions still remain in those reported strategies. Therefore, development of fluorescent probes specifically toward detecting Cu^{2+} in aqueous solution and under physiological relevant conditions is still of great necessity.

Nowadays, several signaling mechanisms have been widely used in chemosensors, such as photo-induced electron/ energy transfer,[25-27] intramolecular charge transfer[28-30] and fluorescence resonance energy transfer.[31-33] Among the various fluorescence signaling mechanisms, isomerization[34-36] based on the fact that C=N isomerization signaling mechanism has already been utilized in sensors,[37-42] while N=N isomerization[43-45] with better quenching effect is still unexplored precisely draws our special attention. Meanwhile, coumarin is a popular fluorescent dye with widespread applications particularly as an ideal fluorophore in the designing of chemosensors due to its superior photophysical and spectroscopic properties, such as long absorption and emission wavelengths, high fluorescence quantum yields and good stability.[46, 47]

Actually, a number of fluorescent probes working on coumarin for detection of metal ions have been reported based on isomerization,[48-51] while most of them exploited the inhibition of C=N isomerization by metal ions' binding-induced conformational restriction rather than formation of covalent bridging of C=N bond, which is more stable. Meanwhile, rare fluorescent probes for Cu^{2+} based on N=N isomerization have ever emerged. Therefore, in this paper, we put forward a novel strategy of suppression of N=N isomerization by Cu^{2+}-catalyzed cyclization from 2-aminoazobenzene to triazole to devise a turn-on fluorescent probe (**A**) for Cu^{2+} (Scheme 1). The new



Scheme 1 The structure of probe **A** and proposed sensing mechanism of Cu^{2+} detection

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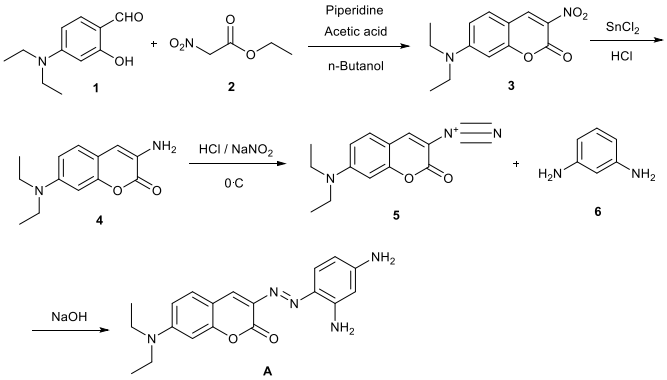
† Electronic Supplementary Information (ESI) available: Detailed synthetic procedures, characterization and ^1H NMR, ^{13}C NMR and HRMS spectra of compounds, and additional cell images. See DOI: 10.1039/x0xx00000x

coumarin derivative **A** was synthesized on the basis of the route shown in Scheme 2, containing an unbridged N=N bond which can reversibly isomerize between *trans* and *cis* configurations at the cost of consuming energy of the molecule, resulting in no fluorescent emission. Whereas upon being exposed to Cu^{2+}, N=N isomerization was suppressed by selective catalytic cyclization to triazole,[52, 53] therefore probe **A** displayed a remarkable green florescence enhancement along with a visible color change from pink to yellow in an aqueous acetonitrile solvent. And it was proved to be an efficient probe selectively for Cu^{2+} over other metal ions at nanomolar level. As a key facet, it can be successfully utilized in detecting Cu^{2+} in living cells.



Scheme 2 Synthetic route to probe **A**

To the best of our knowledge, this is the first fluorescence turn-on probe based on coumarin for Cu^{2+} detection by bridging N=N bond covalently to block its isomerization, which is triggered by Cu^{2+}-catalyzed cyclization to trizole. The performance of probe **A** for Cu^{2+} sensing was compared with other representative turn-on probes for Cu^{2+} reported recently based on various mechanisms[24, 54-59] in Table S1. As shown in it, the proposed probe exhibits excellent analytical performance and therefore is a potent turn-on fluorescent probe for Cu^{2+} monitoring. And it also opens up a new path to applying N=N isomerization to designing fluorescence turn-on probes.

Experimental

Materials

All reagents in the syntheses were purchased from *J & K* (Beijing, China) and used without purification unless otherwise. Solvents were dried by standard methods prior to use. Human liver carcinoma cell line HepG2 was purchased from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China).

Instruments

^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian spectrometer (300 MHz and 75 MHz, respectively). HRMS were recorded on a Bruker maXis 4G mass spectrometer. All pH measurements were made on a Model PHS-3C pH meter. UV-Vis absorption spectra were measured on a TU-1901 spectrophotometer. Fluorescence spectra were taken on a PerkinElmer LS-55 spectrofluorometer. Fluorescence imaging was performed on a Nikon ECLIPSE TE2000-S inverted fluorescence microscope.

Synthesis of probe A

In refluxing n-butanol, 4-diethylamino salicyldehyde was coupled with 1.2 equiv. of ethyl nitroacetate via a one-pot reaction for 24 h to afford the product **3** in 73% yield. Subsequently, **3** was reduced to amino product **4** in the presence of stannous chloride at 85 °C for 4 h. And the final compound **A** (63% yield) was obtained by a one-pot reaction which involved two continuous steps, diazotization and coupling, and be sure that the reaction proceeded under 0 °C all along. The structure of **A** was confirmed by HRMS, ^{1}H NMR, and ^{13}C NMR. HRMS: Mr calculated for $C_{19}H_{21}N_5O_2$, 351.17, found m/z 352.17 $[M + H]^+$. 1H NMR ($CDCl_3$, 300 MHz): δ 1.23 (t, J = 6.9 Hz, 6 H), 3.43 (q, J = 7.2 Hz, 4 H), 5.89 (d, J = 2.4 Hz, 1 H), 6.16 (dd, J = 6.3 Hz, 1 H), 6.55 (d, J = 2.4 Hz, 1 H), 6.61 (dd, J = 6.3 Hz, 1 H), 7.38 (d, J = 8.7 Hz, 1 H), 7.60 (d, J = 8.7Hz, 1 H), 7.92 (s, 1 H), without four active hydrogens showed on it. ^{13}C NMR ($CDCl_3$, 75 MHz): δ 12.27, 12.36, 14.03, 54.86, 59.71, 96.21, 96.50, 106.11, 108.19, 109.57, 122.62, 130.45, 130.57, 132.97, 146.37, 46.56, 149.87, 153.03, 155.11, 159.87.

Solution preparation

Probe **A** was dissolved in acetonitrile to make a 100 μM stock solution, which was further diluted to 10 μM (in spectroscopic measurements) and 5 μM (in cellular imaging) in a mixture of phosphate buffer saline (PBS, 0.01 M, pH 7.4) and acetonitrile (9: 1, v/ v) for subsequent measurements. Stock solutions of Cu^{2+} and other ions were prepared in PBS (0.01 M, pH 7.4) and diluted to the required concentrations for following experiments. UV-Vis absorption and fluorescence measurements were carried out at room temperature.

Cell culture and imaging

HepG2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 units/ mL penicillin, 100 μg/ mL streptomycin) in a humidified atmosphere of CO_2/ air (5: 95%) at 37 °C. Prior to imaging experiments, HepG2 cells were seeded in 12-well plates and cultured for 24 h.

For labeling, the medium was removed and cells were rinsed three times with PBS. Then HepG2 cells were incubated with probe **A** (5 μM) in a mixture of PBS and acetonitrile (9: 1, v/ v) at 37 °C for 30 min as control. For Cu^{2+} imaging, another set of HepG2 cells were preloaded with probe **A** (5 μM) in a mixture of PBS and acetonitrile (9: 1, v/ v) at 37 °C for 30 min, rinsed three times with PBS and further treated with different concentrations of Cu^{2+} (5 μM, 20 μM and 50 μM) in PBS at 37 °C for additional 30 min. Cells rinsed three times with PBS and bathed in it, imaging was carried out. Images were acquired using an inverted fluorescence microscope and fluorescence imaging was conducted in green channel.

Results and discussion

Probe **A** was achieved by diazo-couple reaction from compound **4** in a yield of 63% (Scheme 1). Compound **4** was obtained via the reduction of compound **3**, which was prepared from coupling 4-diethylamino salicyldehyde with ethyl nitroacetate. All compounds were characterized by HRMS, ^{1}H NMR, and ^{13}C NMR.

Spectroscopic properties of probe A and its response to Cu^{2+}

Spectroscopic properties of probe **A** and its response to Cu^{2+} were investigated in a mixture of phosphate buffer saline (PBS, 0.01 M, pH 7.4) and acetonitrile (9: 1, v/ v) at room temperature. Both the UV-Vis absorption and fluorescence spectra are depicted in Fig. 1. It was found that the maximum absorption wavelength of probe **A** (10μM) shifted from 421 nm to 538 nm with the addition of $Cu(CH_3COO)_2$ (100μM) (Fig. 1a), and the fluorescence of the solution increased dramatically with the maximum emission wavelength exhibiting a red-shift from 497 nm to 510 nm due to the formation of the new compound trizole **B** (confirmed by HRMS in Fig. S9, Fig. 1b). Actually, an obvious color change from pink to yellow (Fig. 1a, inset) and fluorescence emission from none to green (Fig. 1b, inset) of the solution can be easily observed by the naked eye.

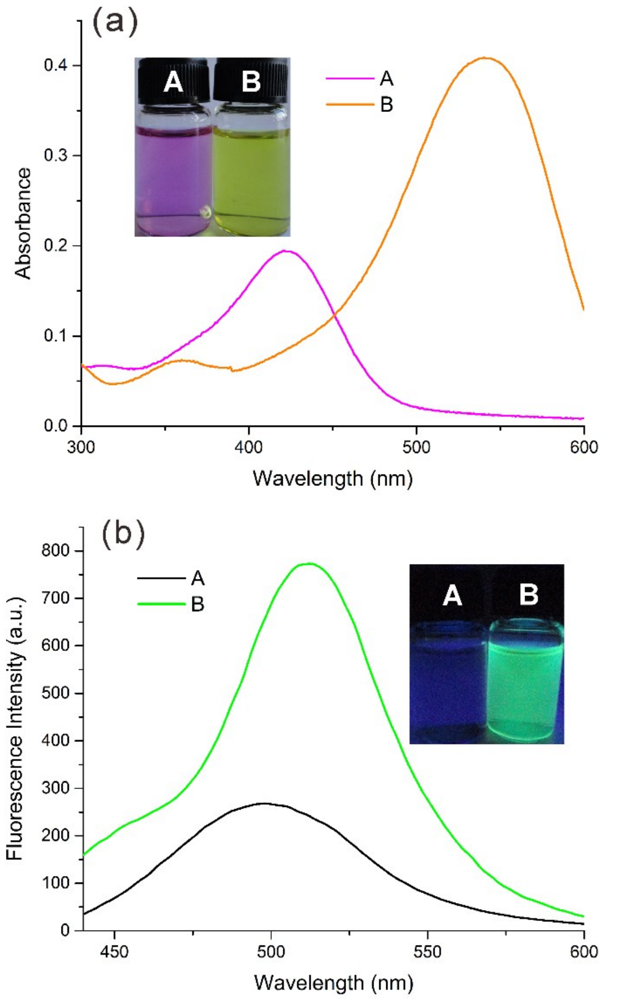


Fig. 1 UV-Vis absorption spectra (a) and fluorescence emission spectra (b) of probe **A** (10 μM) and **B** obtained from **A** (10 μM) reacting with Cu^{2+} (100 μM) in a mixture of PBS (0.01 M, pH 7.4) and CH_3CN (9: 1, v/ v). Excitation wavelength was set at 420 nm for (b). Insets: images of **A** and **B** prepared using the same experimental conditions under room light (a) and under a UV lamp at 365 nm (b).

Fluorescence titration and sensitivity

From the fluorescence titration experiments (Fig. 2a), we can see on addition of increasing concentrations of Cu^{2+} (0 – 80 μM) to the solution of probe **A** (10 μM), fluorescence emission at λ = 510 nm gradually arose, and fluorescence intensity ratio (I/ I_0) at λ = 510 nm also presented a gradual increase as can be found from the inset. The equivalent of Cu^{2+} (10 μM) was sufficient to complete the reaction, displaying the splendid sensitivity of probe **A** for Cu^{2+}. Furthermore, the detection limit of probe **A** was estimated to be 0.02 μM, which is satisfied for Cu^{2+} detection in drinking water within the US EPA limit (~ 20 μM). As can be seen in Fig. 2b, there was a linear correlation between fluorescence intensity ratio (I/ I_0) at λ = 510 nm and Cu^{2+} concentration ranging from 0.02 μM to 5 μM, which made the quantitative determination of Cu^{2+} at a low level in aqueous solution feasible.

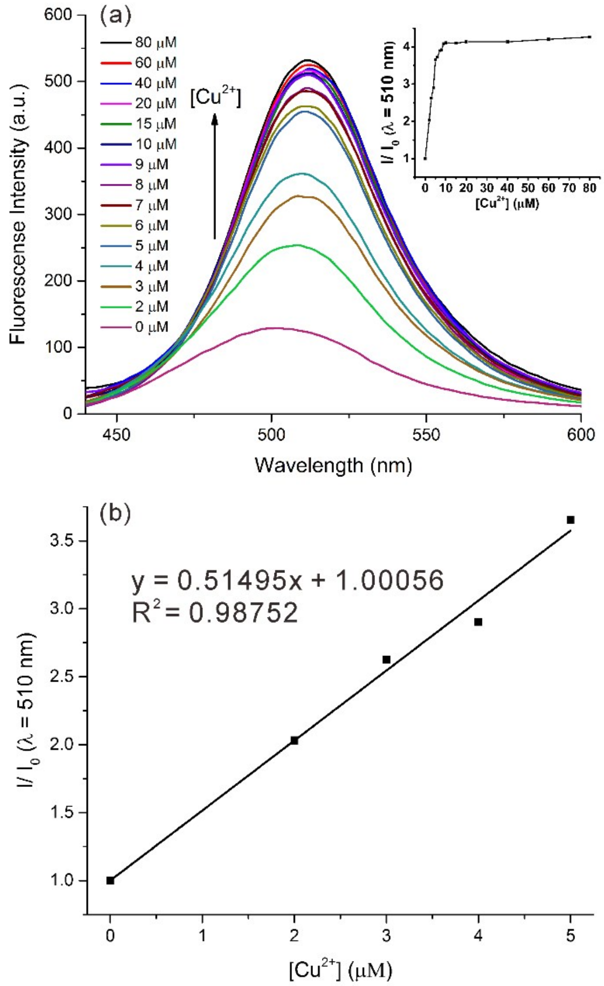


Fig. 2 (a) Fluorescence emission spectra of probe **A** (10 μM) upon addition of increasing concentrations of Cu^{2+} (0 – 80 μM) in a mixture of PBS (0.01 M, pH 7.4) and CH_3CN (9: 1, v/ v). Inset: the plot of fluorescence intensity ratio (I/ I_0) at λ = 510 nm *vs.* Cu^{2+} concentration. (b) The linear correlation between fluorescence intensity ratio (I/ I_0) at λ = 510 nm and Cu^{2+} concentration (0.02 – 5 μM). λ_{ex} = 420 nm.

Kinetics and pH effects

In addition, we evaluated the time course of fluorescent response of probe **A** (10 μM) to Cu^{2+} (50 μM). As shown in Fig. 3a, the reaction of **A** and Cu^{2+} went rapidly, which can be completed within 5 min. Therefore, probe **A** absolutely could be applied in real-time determination of Cu^{2+} in environmental and biological conditions. For practical application, we also evaluated the appropriate pH condition for probe **A**. In acid-base titration experiments, probe **A** (10 μM) reacting with Cu^{2+} (50 μM) to produce **B** remained unaffected at pH 3 - 10 with presenting almost equivalent fluorescence emission (Fig. 3b), suggesting that probe **A** could operate at a wide pH range. In other words, we successfully provided an ideal florescent probe for Cu^{2+} detection under approximate physiological conditions.

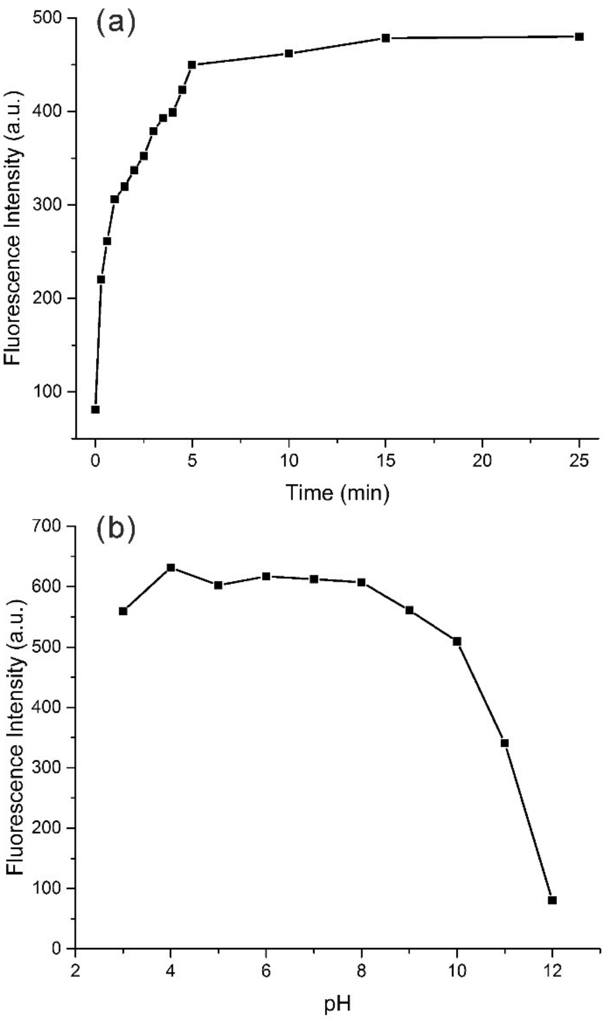


Fig. 3 Time-dependent (a) and pH-dependent (b) changes of fluorescence intensity at λ = 510 nm of probe **A** (10 μM) in the presence of Cu^{2+} (50 μM) in a mixture of PBS (0.01 M, pH 7.4) and CH_3CN (9: 1, v/ v). Different pH values were adjusted by NaOH and HCl aqueous solution. λ_{ex} = 420 nm.

Selectivity of probe A toward Cu^{2+}

One of the most indispensable qualities of a fluorescent probe is the high selectivity toward the specific analyte over other relevant species. To evaluate the selectivity of probe **A** (10 μM) for Cu^{2+} (30 μM), a variety of metal ions (30 μM) including Ag^+, Al^{3+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Fe^{2+}, Fe^{3+}, K^+, Li^+, Mg^{2+}, Mn^{2+}, Na^+, Ni^+, Pb^{2+} and Zn^{2+} were examined under the same condition with Cu^{2+}. As shown in Fig. 4, only addition of Cu^{2+} resulted in a dramatic fluorescence enhancement of the solution of probe **A**, while other metal ions did not lead to obvious fluorescence. After all, we induced Na_2S equivalent to Cu^{2+} to the above fluorescent solution to remove Cu^{2+} by precipitating Cu_2S, and fluorescence intensity was still unchanged, reflecting that the reaction preferred catalysis rather than chelation. Furthermore, the performance of probe **A** in Cu^{2+} detection in the presence of other competitive species was also tested. The competition experiments (Fig. 4) showed that these metal ions displayed no evident interference in the fluorescence increase caused by Cu^{2+}. All the above results indicate that the selectivity of probe **A** for Cu^{2+} in aqueous system is remarkably high.

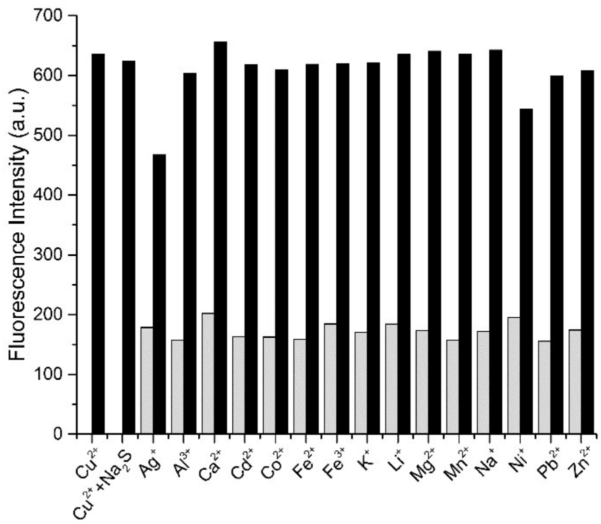


Fig. 4 Fluorescence responses of probe **A** (10 μM) toward kinds of metal ions (30 μM) respectively in a mixture of PBS (0.01 M, pH 7.4) and CH_3CN (9: 1, v/ v) at λ_{em} = 510 nm in the absence (grey bars) and presence (black bars) of Cu^{2+} (30 μM). The second bar represents fluorescence intensity of the reaction system of probe **A** and Cu^{2+} on subsequent addition of Na_2S (30 μM). λ_{ex} = 420 nm.

Cell imaging

Due to the outstanding spectroscopic and chemical properties of probe **A**, we further investigated its biological application in living cells. As is known to all, Cu^{2+} accumulates in liver, therefore, we chose human liver carcinoma cell line HepG2 as the target. HepG2 cells were first incubated with only probe **A**

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(5 μM) in a mixture of PBS (0.01 M, pH 7.4) and acetonitrile (9: 1, v/ v) for 30 min, which led to barely intracellular florescence as determined by inverted fluorescent microscope (Fig. 5b). Then the cells preloaded with probe **A** (5 μM) were further incubated with Cu^{2+} (50 μM) in PBS for 30 min, whereupon a significant increase of fluorescence inside the cells was observed (Fig. 5c). Bright-field measurements confirmed that the cells were viable throughout the experiments (Fig. 5a). Bioimaging of lower concentrations of Cu^{2+} (5 μM and 20 μM) was also realized (Fig. S11). These results indicate that probe **A** is low cytotoxic, cell membrane permeable and capable of imaging Cu^{2+} in living cells.

Fig. 5 Fluorescence imaging of probe **A** with Cu^{2+} in HepG2 cells. (a) Bright-field image of HepG2 cells preloaded with probe **A** (5 μM) in a mixture of PBS (0.01 M, pH 7.4) and acetonitrile (9: 1, v/ v) for 30 min and then incubated with Cu^{2+} (50 μM) in PBS for additional 30 min at 37 °C. (b) Fluorescence image in green channel of HepG2 cells treated with **A** (5 μM) for 30 min at 37 °C. (c) Fluorescence image in green channel of HepG2 cells preloaded with probe **A** (5 μM) for 30 min and then incubated with Cu^{2+} (50 μM) for additional 30 min. Scale bar represents 100 μm.

Conclusions

In summary, we have designed and synthesized a novel coumarin-based fluorescence turn-on probe **A** for Cu^{2+} with signaling mechanism of N=N isomerization, which can be utilized in aqueous solution at a wide pH range. The probe features a rapid response to Cu^{2+} with an evident fluorescence enhancement owing to the formation of the new compound triazole. Remarkably, probe **A** shows excellent sensitivity and selectivity for Cu^{2+} over other metal ions. Significantly, the intracellular Cu^{2+} imaging capacity in HepG2 cells further demonstrates that probe **A** is a valuable tool in living systems.

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View Article Online
DOI: 10.1039/C5NJ03649F

Graphical abstract

A "turn-on" probe **A** based on coumarin with mechanism of N=N isomerization was developed for detecting Cu^{2+} in living cells.