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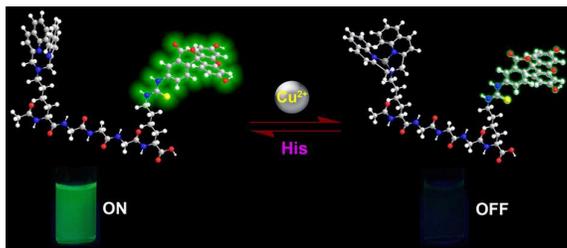
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3 A new fluorescence switch was developed for sequentially and selectively sensing Cu^{2+} and L-Histidine (L-His) *in*
4 *vitro* and in living cells.
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COMMUNICATION

Fluorescence switch for sequentially and selectively sensing copper (II) and L -histidine in vitro and in living cells†

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Herein, we report the development of a new fluorescence switch for sequentially and selectively sensing Cu^{2+} and L -Histidine (L -His) in vitro and in living cells for the first time. In the absence of metal ions, Ac-SAACQ-Gly-Gly-Gly-Lys (FITC) (1) exhibits comparable fluorescence to that of free FITC. In the presence of metal ions, 1 selectively coordinates with Cu^{2+} , causing its fluorescence emission quenched via photoinduced electron transfer. Interestingly, as-formed 1- Cu^{2+} complex selectively responds to L -His among the 20 natural amino acids by turning its fluorescence on. This property of fluorescence switch of 1 was successfully applied to qualitatively and quantitatively sensing Cu^{2+} and L -His in vitro. Using this dualfunctional probe, we also sequentially imaged Cu^{2+} and L -His in living HepG2 cells. Our new probe 1 could be applied for not only environment monitoring but also biomolecule detections in the near future.

Copper ion (Cu^{2+}) plays a key role in many physiological processes including gene expression and protein functioning,¹ and is also required by human nervous system.² Copper deficiency is associated with myelopathy³ but high levels of Cu^{2+} may lead to the damages of liver or kidney.⁴ Up to date, a tremendous number of methods have been developed for the detection of copper.⁵ For example, traditional instrument-assisted technologies such as inductively coupled plasma mass spectroscopy (ICPMS)⁶ and atomic absorption/emission spectroscopy (AAS/AES)⁷ have been applied for the detection of Cu^{2+} as standard methodologies. The newly developed instrument-assisted techniques based on surface plasmon resonance (SPR)⁸ or surface-enhanced Raman scattering (SERS)⁹ were also successfully applied for the detection of Cu^{2+} with high sensitivity. However, the requirement of sophisticated, expensive equipment, and highly trained personnel of the aforementioned techniques restricts their application in the facile and practical detection of Cu^{2+} . This

calls for new methods and many small molecular-based (SMB) systems had been developed for this purpose. For example, new “off” (or “on”) fluorescent probes¹⁰ or nucleic acid sensors¹¹ have been developed for the detection of Cu^{2+} with high sensitivities. Nevertheless, there are still some limitations in these systems including tedious procedures of sample preparation, rigid reaction conditions, low accuracies due to the chemical/physical interference from similarities of coexisting metal ion species,¹² aggregations of the probes at micromolar concentrations,¹⁰ⁱ or poor biocompatibility^{10j}.

L -Histidine (L -His, HisH) in the form of free amino acid (AA) acts as a tridentate ligand toward transition-metal ions.¹³ Since the discovery of Cu^{2+} - L -His species in human blood and their treatment for Menkes disease (a fatal genetic neurodegenerative disease), a great deal of interest has been generated.¹⁴ Recently, using Cu^{2+} - L -His to treat infantile hypertrophic cardioencephalomyopathy has also been reported.¹⁵ To date, there are extensive research efforts that have been made to determine the role of L -His in cellular uptake of copper. Nevertheless, few sensors have been developed for a successful detection of L -His due to their either poor selectivity or low limit of detection to L -His among the 20 natural AAs.¹⁶

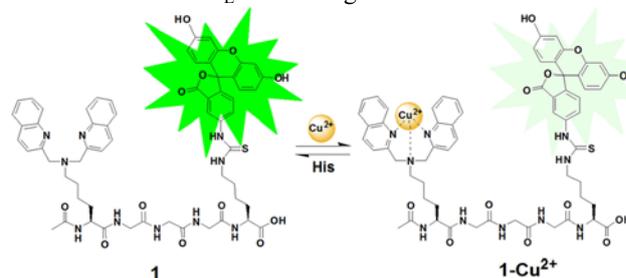


Fig. 1 Schematic illustration of a fluorescence switch for the sequential detections of Cu^{2+} and L -His

Encouraged by these, as shown in Fig. 1, we designed a fluorescence switch using a water soluble probe Ac-SAACQ-Gly-Gly-Gly-Lys(FITC)-OH (**1**) for the sequential detections of Cu^{2+} and L-His in vitro and in living cells with high selectivity for the first time. The Cu^{2+} -binding motif used in this work is a lysine-derived quinoline, referred as a single AA chelate quinoline (SAACQ), firstly reported by Valliant and his co-workers.¹⁷ Unlike those fluorescence probes which directly conjugate with Cu^{2+} resulting in fluorescence "off" for irreversible detection of Cu^{2+} , we separated the Cu^{2+} -binding motif (i.e., SAACQ) from the fluorophore (herein FITC) due to the following two reasons. Firstly, the separated SAACQ- Cu^{2+} complex still can quench the fluorescence of FITC via photo-induced electron transfer (PET).¹⁸ Secondly, the separated SAACQ- Cu^{2+} complex has good accessibility to L-His . Thus, the subsequent competitive binding of Cu^{2+} with L-His leads to the disassociation of the SAACQ- Cu^{2+} complex, resulting in the fluorescence recovery of **1** for L-His detection. Between the SAACQ- Cu^{2+} complex and FITC, we put Gly-Gly-Gly-Lys (GGGK) as the shortest linker to ensure the water solubility and biocompatibility of **1** while in the meantime retain its PET effect. Moreover, since the SAACQ can be regarded as a natural AA, the Ac-SAACQ-Gly-Gly-Gly-Lys-OH backbone for **1** could be synthesized in one-step with solid phase peptide synthesis (SPPS) (Fig. S1, S3 and Scheme S1, ESI†). Interestingly, we found this water soluble, dualfunctional probe **1** is selectively sensitive to Cu^{2+} and L-His , suggesting its importance for biomolecule detections.¹⁹

The aforementioned probe **1** contains two segments: the non-fluorescent Ac-SAACQ-Gly-Gly-Gly-Lys-OH (**1b**) backbone for Cu^{2+} chelation and the fluorophore fluorescein isothiocyanate (FITC) (Fig. 1 and Scheme S2, ESI†). Compared with free FITC in the same condition, our probe **1** exhibited 69.6% fluorescence intensity (FI) of that of free FITC (3373 vs. 4844, Fig. S4, ESI†), suggesting the modification of FITC with **1b** do not induce an obvious decrease of the fluorescence intensity (FI) of the fluorophore. Upon addition of $20 \mu\text{M}$ Cu^{2+} at room temperature (RT), both spectroscopic and photographic fluorescence of $10 \mu\text{M}$ **1** in Tris-HCl buffered (pH 7.5, 50 mM) solution dramatically decreased (Fig. 2 and Fig. S5, ESI†). To ensure the FI decrease of **1** was resulted from the coordination between Cu^{2+} and SAACQ instead of the direct binding of Cu^{2+} to FITC, we measured the UV-vis and fluorescence spectra of **1b**, FITC, and **1** before and after Cu^{2+} addition. The results showed that addition of Cu^{2+} did not induce obvious change of either the UV-vis or the fluorescence spectra of FITC, but did induce changes of both of **1** and changes of the UV-vis spectra of **1b** (Fig. S6, ESI†). This indicated that it is the coordination of Cu^{2+} with SAACQ on **1** that stabilizes the metal ion in the proximity of fluorophore FITC, thus quenches the fluorescence of FITC.

In addition to the 6 nm blue-shift of its UV-vis spectra at 498 nm of **1** after Cu^{2+} addition (Fig. S6, ESI†), we also conducted ¹HNMR and electrospray ionization mass (ESI-MS) spectroscopic analyses to confirm the coordination between SAACQ and Cu^{2+} . For ¹HNMR spectroscopic analysis,

fluorenylmethoxycarbonyl-SAACQ (Fmoc-SAACQ) as model compound was used. Since Cu^{2+} is a paramagnetic ion which heavily interferes with ¹HNMR signals, we then chose Zn^{2+} as an alternative of Cu^{2+} to study its coordination with Fmoc-SAACQ.²⁰ ¹HNMR spectra of Fmoc-SAACQ showed that the proton resonances on the quinoline rings were shifted downfield and became broader upon the addition of 2 equiv of ZnCl_2 , indicating the coordination of Zn^{2+} to Fmoc-SAACQ (Fig. S7, ESI†). This indicates that the coordination reduces the conformational degrees of freedom of the two quinoline moieties and inhibits their intramolecular π - π stacking. ESI-MS spectra of **1** upon addition of Cu^{2+} clearly showed that there are two major peaks of m/z 1222.34 and 611.62, which have typical isotopic peaks similar to those of Cu^{2+} , corresponding to $[\mathbf{1-Cu}]^+$ and $[\mathbf{1-Cu}]^{2+}$ respectively (Fig. S8, ESI†). These above results echoed that the FI decrease of **1** upon Cu^{2+} addition was induced by the coordination between the copper ions and quinoline moieties and therefore the FI of FITC was quenched via PET mechanism. Under these conditions, a 1:1 probe cooper stoichiometry is assumed, and binding constant (K_s) of probe **1** with Cu^{2+} was calculated to be $1.5 \times 10^5 \text{ M}^{-1}$ (Fig. S9, ESI†). It was reported the binding constant of L-His towards Cu^{2+} is 1×10^8 - 1.3×10^{10} (Table S2),^{13c} much larger than that of our probe **1**. Thus, using the **1-Cu}^{2+} complex for the following L-His detection becomes feasible. On the other hand, it is infeasible for **1** to detect the Cu^{2+} which is already pre-complexed with L-His .**

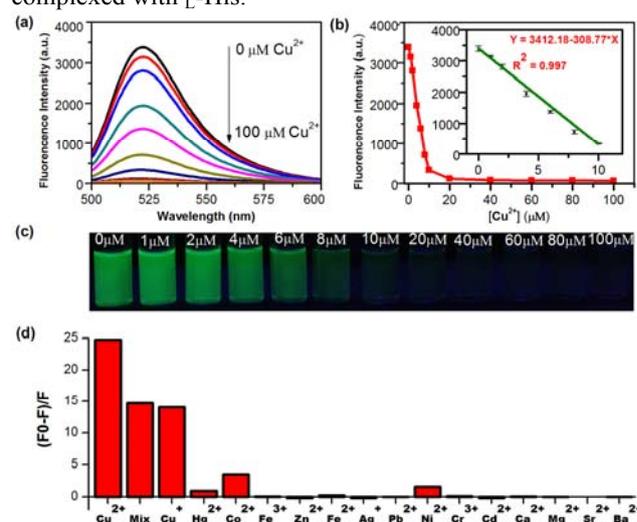


Fig. 2 (a) Fluorescence spectra of probe **1** ($10 \mu\text{M}$, $\lambda_{\text{ex}} = 465 \text{ nm}$) in the presence of various concentrations of Cu^{2+} (0, 1, 2, 4, 8, 10, 20, 40, 60, 80, and $100 \mu\text{M}$) in Tris-HCl buffered (pH 7.5, 50 mM) aqueous solution at RT. (b) Correlation of $\Delta FI_{521 \text{ nm}}$ in part a with Cu^{2+} concentration between 0 and $100 \mu\text{M}$. The inset is a fitted calibration line in the linear region of 0- $10 \mu\text{M}$ Cu^{2+} . The error bar represents the standard deviation of three measurements. (c) The corresponding fluorescence photos of **1** in a cuvette in the presence of different concentrations of Cu^{2+} under a UV lamp. (d) Competitive selectivity of $10 \mu\text{M}$ **1** in the presence of Cu^{2+} (2 equiv.), mixture, 2 equiv. of Cu^+ , Hg^{2+} , Co^{2+} , Fe^{3+} , Zn^{2+} , Fe^{2+} , Ag^+ , Pb^{2+} , Ni^{2+} , Cr^{3+} , Cd^{2+} , Ca^{2+} , Mg^{2+} , Sr^{2+} , Ba^{2+} respectively. Emission: 521 nm.

Fig. 2a shows the fluorescence spectra of 10 μM **1** before and after the addition of different concentrations of Cu^{2+} at RT in Tris-HCl buffered (pH 7.5, 50 mM) solution for 5 min. In general, the fluorescence of **1** decreases with the increase of Cu^{2+} concentration. The fluorescence photos of **1** in a cuvette under a UV lamp, corresponding to these changes, are shown in Fig. 2c. By correlating the fluorescence change at 521 nm (i.e., $F_0 - F$, $\Delta F_{521 \text{ nm}}$) with the concentration of Cu^{2+} , we obtained a calibration curve for the determination of Cu^{2+} in vitro. As shown in Fig. 2b, a linear relationship between the $\Delta F_{521 \text{ nm}}$ and Cu^{2+} concentration ($Y = 3412.18 - 308.77X$, $R^2 = 0.997$) was obtained over the range of 0-10 μM . The limit of detection (LOD) of Cu^{2+} of this assay is 0.24 μM ($S/N = 3$) which is satisfactorily below the limit of Cu^{2+} in drinking water settled by U.S. Environmental Protection Agency (EPA) ($\sim 20 \mu\text{M}$),^{5c} but far exceeds the concentration of free copper ion in an unstressed cell (about 10^{-18}M)²¹.

Selectivity is one of the important parameters to evaluate the performance of a new fluorescence probe. Particularly, for a cellular imaging probe which potentially has biomedical or environmental applications, a highly selective response to the target over other potentially competing species is a necessity. Therefore, the selectivity study of **1** to Cu^{2+} over various metal ions such as abundant cellular cations (e.g., Ca^{2+} and Mg^{2+}), essential metal ions in cells (e.g., Co^{2+} , Fe^{3+} , Zn^{2+} , Fe^{2+} , Ni^{2+} , Sr^{2+} , Ba^{2+}), and environmentally relevant heavy metal ions (e.g., Hg^{2+} , Ag^+ , Pb^{2+} , Cr^{3+} , Cd^{2+}), was conducted. As shown in Fig. 2d, mixing the 10 μM **1** with 20 μM Cu^{2+} induced a fluorescence change ratio of **1** at 521 nm (i.e., $(F_0 - F)/F$) as large as 24.8. However, separately mixing 10 μM **1** with other abovementioned metal ions at 20 μM did not induce an obvious FL change ratio (the highest is 3.5 for Co^{2+} except Cu^+). Interestingly, mixing 10 μM **1** with all the metal ions studied (including Cu^{2+}) at 20 μM induced a FI change ratio of 14.6, suggesting **1** has a good selectivity to Cu^{2+} even under the heavy interference of other fourteen metal ions. These excellent features of **1** warrant its potential applications in environmental monitoring and biomolecule detections.

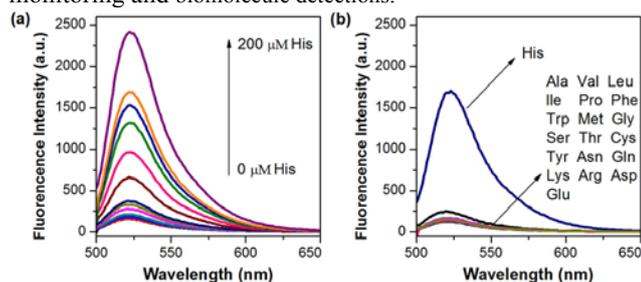


Fig. 3 (a) Fluorescence spectra of **1-Cu**²⁺ complex (10 μM **1** and 10 μM Cu^{2+}) upon addition of various concentrations of L-His (0, 1, 2, 4, 8, 10, 20, 40, 60, 80, 100, and 200 μM) in Tris-HCl buffered aqueous solution (pH 7.5, 50 mM) at RT. The fitted calibration line in the liner region of 1-60 μM of L-His (Fig S10, ESI[†]). (b) Selectivity study of **1-Cu**²⁺ complex (10 μM **1** and 20 μM Cu^{2+}) to L-His among the twenty natural AAs (100 μM for each).

Since L-amino acids (L-AAAs) play a key role in the diagnosis and treatment of disease, many efforts have been made to

develop feasible approaches for their detections.^{13a, 22} Among the 20 natural AAs, L-His is very important and well known for its chelation property for metal ions such as Ni^{2+} . Therefore, in this study, we also applied **1** for sequential detection of L-His after its coordination with Cu^{2+} . As shown in Fig. 3a, after the formation of **1-Cu**²⁺ complex, progressive addition of L-His into the system gradually turned the fluorescence “on”. This indicated that L-His competitively binds with the Cu^{2+} in the **1-Cu**²⁺ complex by forming a more stable complex $\text{Cu}^{2+}\text{-L-His}$, freeing **1** from the complex and thus recovering its fluorescence. By correlating the fluorescence change ratio at 521 nm (i.e., $(F - F_0)/F_0$) with the concentration of L-His , we obtained a calibration curve for the determination of L-His in vitro. As shown in Fig. S10, a linear relationship between the $(F - F_0)/F_0$ and L-His concentration ($Y = -0.22301 + 0.13376X$, $R^2 = 0.959$) was obtained over the range of 1-60 μM . The limit of detection (LOD) of L-His of this assay is 1.6 μM ($S/N = 3$). Surprisingly, the fluorescence of **1-Cu**²⁺ complex could only be turned “on” by L-His among the 20 natural AAs, suggesting a great selectivity of this system for L-His detection (Fig. 3b).

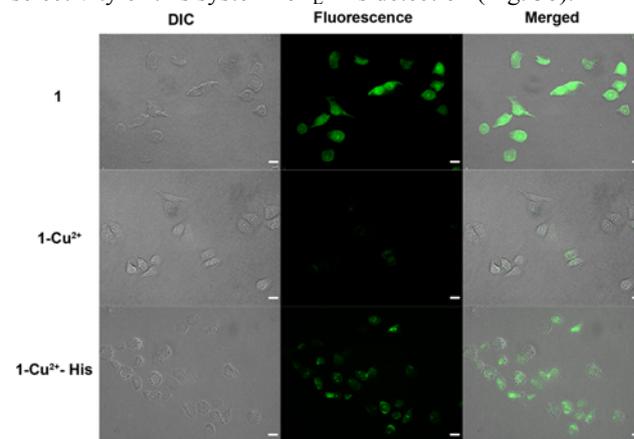


Fig. 4 Differential interference contrast (DIC) images (left), fluorescence images (middle, EGFP channel), and merged images (right) of HepG2 cells incubated with 10 μM of probe **1** in serum-free medium for 1 h at 37 $^{\circ}\text{C}$, washed with PBS for three times prior to imaging (upper row), incubated with 20 μM Cu^{2+} in serum-free medium for 20 min at 37 $^{\circ}\text{C}$ prior to imaging (middle row), then incubated with 200 μM L-His in serum-free medium for 20 min at 37 $^{\circ}\text{C}$ prior to imaging (lower row), respectively. Scale bar: 20 μm .

The excellent property of fluorescence switch of **1** was also successfully applied to sequentially imaging Cu^{2+} and L-His in living cells. As shown in the upper row of Fig. 4, after the healthy HepG2 cells were incubated with 10 μM **1** in serum-free culture medium at 37 $^{\circ}\text{C}$ for 1 h and washed three times with phosphate buffered saline (PBS, pH 7.4) to remove the free **1**, bright green fluorescence cell imaging could be observed. This suggests that our water soluble probe **1** has much better cell permeability than FITC and is suitable for living cell imaging (Fig. S11, ESI[†]). After that, 20 μM Cu^{2+} was added into the medium and incubated with the cells for 20 min at 37 $^{\circ}\text{C}$ prior to imaging. Obviously we found the fluorescence emission from the cells was dimmed out (middle row of Fig. 4). The process of this fluorescence “dimming out”

was clearly observed by incubating the cells with increasing Cu^{2+} concentrations (Fig. S12, ESI†). The highest concentration of Cu^{2+} in our cell study (i.e., 20 μM) should be far below that inducing cytotoxicity.²³ Interestingly, after subsequent addition of 200 μM of L-His into the culture medium and the cells were incubated for another 20 min, we found the fluorescence emission from the cells was turned “on” again (low row of Fig. 4). Interestingly, from Fig. 4, we found that the subcellular distribution of **1** in the **1**-treated cells alone was different from that in the **1**- Cu^{2+} - L-His -treated cells, suggesting that **1** is not ratiometric to each individual cell. Additional experiments indicated that the recovery of the fluorescence of **1**- Cu^{2+} complex was not induced by small molecule inside cells such as glutathione (GSH) (Fig. S13, ESI†). The process of the fluorescence “turn-on” was also monitored by incubating the cells with increasing of L-His concentrations (Fig. S16, ESI†).

Conclusions

In conclusion, we have successfully developed a new fluorescence switch for sequential and selective detections of Cu^{2+} and L-His in vitro and in living cells. The new water-soluble and cell-permeable probe **1** has a SAACQ motif for Cu^{2+} chelation and its fluorescence from the fluorophore FITC can be quenched via PET mechanism after Cu^{2+} chelation. This property was successfully applied for highly selective Cu^{2+} detection within the range of 0–10 μM and a LOD of 0.24 μM . Interestingly, formed **1**- Cu^{2+} complex could be applied for selective detection of L-His within 1–60 μM among the twenty natural AAs with a LOD of 1.6 μM . With the excellent property of **1** for the detections of Cu^{2+} and L-His with high selectivity, we also successfully applied it for sequentially imaging Cu^{2+} and L-His in living cells. We hope that our new dualfunctional probe **1** could be applied for not only environment monitoring but also biomolecule detections in the near future.²⁴

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Notes and references

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† Electronic Supplementary Information (ESI) available: Experiment methods and details, syntheses and characterizations of Fmoc-SAACQ, **1a**, **1b**, and **1**, HPLC conditions, UV-vis, Fluorescence, NMR, cell imaging, supplementary

schemes, tables, and figures (Schemes S1–S2, Table S1, Figures S1–S17). See DOI: 10.1039/b000000x/

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