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A coumarin-based fluorescent chemosensor CAQA has been synthesized. It can selectively and sensitively recognize Cu^{2+} in aqueous acetonitrile solutions. Using the Cu-containing complex CAQA-Cu^{2+} as a sensing ensemble, the device demonstrates highly selective recognition for His/biothiols and was applied to fluorescence imaging of histidine in hard-to-transfect living cells.

The development of selective and efficient signaling systems to detect biologically relevant cations, anions and small neutral molecules has attracted significant attention in recent years.[1] Fluorescent sensing probes have demonstrated outstanding characteristics in the detection of various analytes such as high selectivity, low detection limits, real-time detection, and high-throughput.[2] Attributed to their intrinsic high fluorescent quantum yield, good water solubility and viability for chemical transformations, coumarin derivatives have attracted much attention as one of the most popular fluorophores amenable to novel sensor design.[3] By judicious incorporating a suitable receptive binding unit onto a coumarin molecular platform, fluorescent metal chemosensors for Cd^{2+}, Cu^{2+}, Hg^{2+}, and Zn^{2+}, have been well developed in the literature.[4-7] Due to its intrinsic paramagnetic property, Cu^{2+} has the propensity to quench the fluorescence of fluorescent metal chelators conferring a non-fluorescence off state in ensemble devices.[8] Subsequent effective snatching of Cu^{2+} ion from the ensemble in aqueous solution by a copper-binding analyte can switch on "turn-on" fluorescence of the sensing ensemble. Working on such two-stage signal transduction mechanism schematically shown in Fig. 1, novel fluorescent probes for biologically or environmentally relevant analytes have been realized.[9-12] There are several advantages associating with this sensing methodology especially for applying in cell imaging process: (1) the water solubility of a metal coordinated complex in an ensemble system can be greatly improved, favouring the cell imaging application; (2) different species sensing can be achieved by simply changing the metal ion of the ensemble;[13] (3) an ensemble oftentimes can be accessible via a simple synthetic route. For instance, exploitation of the paramagnetic fluorescence quenching ability of Cu^{2+}, operative on "On-Off-On" signaling motif, sensing ensemble systems comprising multifunctional fluorophore ligated to Cu^{2+} centre have been developed for selective detection of cyanide,[9] cysteine,[10] histidine,[11] and sulfide.[12]

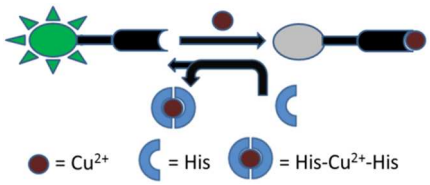
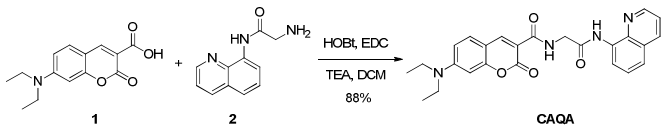


Fig. 1 Illustration of the design and working mechanism of sensing ensemble.

Being an essential amino acid, histidine is indispensable for human growth.[14] It plays vital roles in biological system such as serving as a neurotransmitter[15] and as a controller for metals transmission.[16] Excessive of histidine may cause stress and psychological disorders,[17] whereas the deficiency of histidine may result in the chronic kidney disease[18] and pulmonary disease.[19] Thus, determination of histidine especially in biological samples is an important analytical tool to examine the homeostasis of this species in biological system. To meet this challenge, many analytical methods have been developed such as fluorescent sensors,[11,20] colorimetric detection,[21] electrochemical method[22] and capillary electrophoresis.[23] In this connection, Yu and co-workers very recently developed a novel coumarin-DPA-Cu^{2+} chemosensing ensemble for selective detection of histidine in biological fluids.[11] Prompted by their report, in connection with our continued research interests in fluorescent sensor development for amino acids,[24] we herein present our design of coumarin-based histidine sensing

ensemble which is amenable to selective detection of histidine not only in aqueous solutions but also applicable to living cells. In contrast to the cell permeable fluorescent on-off probe for histidine and cysteine reported by Rao and coworkers,[20b] to our knowledge, our design is the first example of a novel turn-on fluorescent selective probe demonstrating in intracellular detection of histidine in "hard-to-transfect" glioblastoma cells.



Scheme 1 Synthesis of probe **CAQA**.

The synthesis of 7-*N*,*N*-diethylamino**c**oumarin-4-*N*-2-**a**mino-*N*-(**q**uinolin-8-yl) **a**cetamide (**CAQA**) was achieved by coupling of 7-*N*,*N*-diethylcoumarin-4-carboxylic acid (**1**) with 2-amino-*N*-(quinolin-8-yl)acetamide[25] (**2**) promoted by HOBt and EDC (Scheme 1). The structure of **CAQA** was confirmed with ^{1}H NMR, ^{13}C NMR and HRMS spectroscopic methods (Fig. S1-S4, ESI). To confer a selective metal ion chelating property on coumarin derivative **1**, trifunctional metal binding receptive unit **2** was appended onto its 7-carboxyl functionality, affording the highly fluorescent dyad **CAQA**. For tuning the sensing ensemble at a non-fluorescence off-state, a variety of metal ions including Na^+, K^+, Li^+, Ca^{2+}, Mg^{2+}, Fe^{3+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Pb^{2+}, Hg^{2+}, Ag^+ and Cd^{2+} were systematically introduced to the aqueous pH 7.4 ACN-HEPES buffer solution of **CAQA**. By treatment with different metals, the fluorescence of **CAQA** could be extensively quenched only by Cu^{2+} (Φ_F = 0.015, τ = 1.2 ns for **CAQA** and Φ_F < 0.001 for **CAQA**-Cu^{2+} ensemble, quinine sulfate in 0.1 M NaOH as a standard, Φ_F = 0.58),[26] while slightly reduction in fluorescence was caused by Ni^{2+} (Fig. S5, ESI). The selective binding of the probe to Cu^{2+} and Ni^{2+} was also evident by examining the UV-vis spectra of the respective metal complexes. A bathochromic shift of 12 nm and 5 nm was observed for **CAQA**-Cu^{2+}, **CAQA**-Ni^{2+}, respectively in comparison with the apo-ligand **CAQA** (Fig. S6, ESI). To investigate the binding mode between **CAQA** and Cu^{2+}, **CAQA** (5 μM) was titrated with increasing concentration of Cu^{2+}. Fig. 2a and Fig. S7 shows 1 equiv. of Cu^{2+} can switch off the fluorescence of the probe and the fluorescence intensity of **CAQA** was linearly proportional to the concentration of Cu^{2+} ranging from 0 – 3.5 μM (Fig. 2a inset). Job's plot analysis and the MALDI-TOF-MS of the complex clearly reveal that **CAQA** and Cu^{2+} form a 1:1 complex **CAQA**-Cu^{2+} (Fig. S8-S9, ESI). Furthermore, according to the Lineweaver-Burke equation [Equation (1)][27]

$$1/(F_0 - F) = 1/F_0 + K_{LB}/(F_0 \cdot [Q]) \quad (1)$$

where F_0 and F are the steady state fluorescence intensities in the absence and presence of quencher, respectively. [Q] is the concentration of quencher, Cu^{2+}. K_{LB} is the static quenching constant. The linear relationship between $1/(F_0 - F)$ and $1/[Cu^{2+}]$ demonstrated that the static quenching occurs (Fig. 2b). The level of detection (LOD) estimated for Cu^{2+} determination is calculated to be 52 nM (*S*/*N* = 3). Evidently, **CAQA** possessing such a high selectivity and sensitivity could serve as a fluorescent ON-OFF sensor for Cu^{2+}.

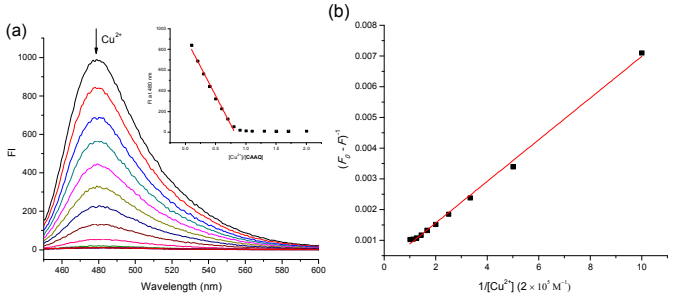


Fig. 2 (a) Fluorescence spectra of **CAQA** (5 μM) in 20% ACN-HEPES (20 mM, pH = 7.4) upon addition of Cu^{2+}. Inset: Plot of fluorescence intensity at 480 nm of **CAQA** (5 μM) versus the concentration of Cu^{2+}. (b) Lineweaver-Burke plot of **CAQA** and Cu^{2+} association in the range of 0-1.0 eq.

We envisage that many biological relevant small molecules such as amino acids can snatch copper from **CAQA**-Cu^{2+}, conducing the recovery of the fluorescence quenched by Cu^{2+} to furnish a turn-on sensor. In practice, **CAQA**-Cu^{2+}, prepared by mixing an equal amount of **CAQA** and $Cu(ClO_4)_2$ (5 μM) in aqueous ACN-HEPES buffer solution, was allowed to treat separately with 20 equiv of twenty essential amino acids, *N*-acetylcysteine (NAC), glutathione (GSH), histamine and imidazole. Fluorescence measurements on the resulting mixtures shown in Fig. 3a reveal that only histidine (His), cysteine (Cys), NAC, homocysteine (Hcy) and GSH can recover the fluorescence of **CAQA** by more than 95%. To probe the binding mode of histidine to Cu^{2+}, as shown in Fig. 3a (Fig. S10 in details), histamine and imidazole were found to be ineffective to trigger any change in the fluorescence of the ensemble. It becomes apparent that the seizure of Cu^{2+} from **CAQA**-Cu^{2+} by histidine could be attributed to the cooperative chelating action of the carboxyl and imidazole moiety of histidine.[28] The 2:1 binding model of His-Cu^{2+} was confirmed by the Job's plot (Fig. S11, ESI). The fluorescence recovery induced by histidine is shown in fluorescent titrations of the ensemble with histidine (Fig. 3b and Fig. S12, ESI). The association constant between histidine and Cu^{2+} was calculated to be 2.54 x 10^9 M^{-2}. In addition, through the UV-vis titrations of **CAQA**-Cu^{2+} with histidine, the full recovery of the UV-vis spectrum of **CAQA** could be realized by the addition of 10 equiv of histidine (Fig. 3c). On the other hand, the ^{1}H NMR spectroscopic method was used to probe the binding mechanism. As shown in Fig. S13-S14, due to the paramagnetic property of Cu^{2+}, all proton resonates of **CAQA** underwent peak broadening when it was mixed with Cu^{2+}. Subsequently addition of histidine into **CAQA**-Cu^{2+} solution caused the resumption of the fine structure of **CAQA** in the ^{1}H NMR spectrum of the mixture. This result further confirmed histidine is capable of removing Cu^{2+} from its **CAQA** complex. Incidentally the fluorescence recovery of the ensemble could also be observed when the sensing ensemble was titrated separately with Cys, Hcy and GSH (Fig. S15-S17, ESI). Knowing that snatching of Cu^{2+} ion from its chelated complexes by histidine and biothiols

was well documented and has been constituted many novel sensing ensemble developments.[11-12]

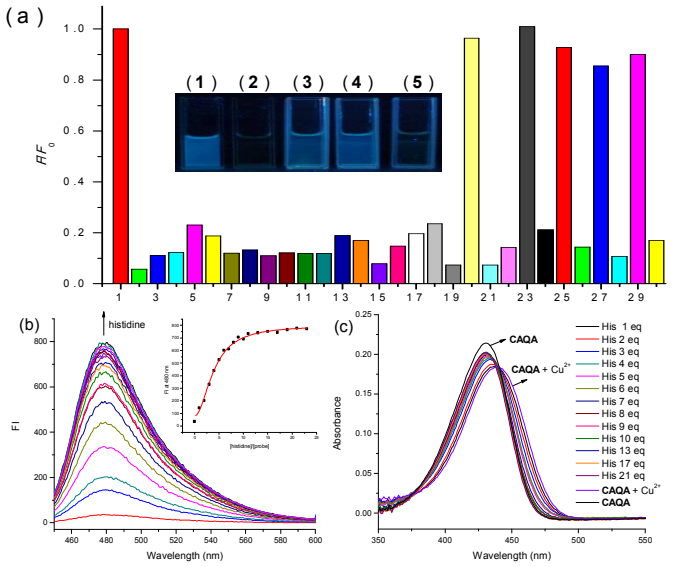


Fig. 3 (a) Fluorescence response ratio of **CAAQ**-Cu^{2+} ensemble (5 μM) towards various analytes: (1) **CAQA** only; (2)**CAQA** + Cu^{2+} ensemble only; (3) Ala; (4) Arg; (5) Asn; (6) Asp; (7) Gln; (8) Glu; (9) Gly; (10) Ile; (11) Leu; (12) Lys; (13) Met; (14) Phe; (15) Pro; (16) Ser; (17) Thr; (18) Trp; (19) Val; (20) His; (21) imidazole; (22) histamine; (23) Cys; (24) Cys + NEM; (25) Hcy; (26) Hcy + NEM; (27) GSH; (28) GSH + NEM; (29) NAC; (30) NAC + NEM. Inset: Change in fluorescence of **CAQA** (20 μM) in the presence of various analytes. From left to right: (1) **CAQA** only; (2) **CAQA** + Cu^{2+}; (3) **CAQA** + Cu^{2+} + His; (4) **CAQA** + Cu^{2+} + biothiols; (5) **CAQA** + Cu^{2+} + biothiols-NEM adduct; (b) Fluorescence and (c) absorption spectra of **CAQA**-Cu^{2+} ensemble (5 μM) in 20% ACN-HEPES (20 mM, pH 7.4) upon the addition of histidine.

It is noteworthy that while fluorescent chemosensor design for detecting biothiols is well developed,[1b] in contrast, selective histidine fluorescent turn-on sensors applicable to cell imaging are scarce. The use of *N*-ethylmaleimide (NEM) as a biothiol scavenger can eliminate the interference of Cys, Hcy, GSH and NAC, which allow us to develop **CAQA**-Cu^{2+} as a selective histidine sensing ensemble.[29] As such, when mixtures of biothiols and NEM were introduced separately into the ensemble, no fluorescent enhancement was observed due to the trapping of sulfhydryl group of biothiols by NEM (Fig. 3a). Detailed interference study results were shown in Fig. S18 (ESI), the present ensemble can be qualified as a highly selective sensing device for histidine. Additionally, the LOD of **CAQA**-Cu^{2+} for the detection of histidine is calculated to be 2.6 x 10^{-7} M as estimated from Fig. 3b. The pH application range of the present sensing system to the detection of Cu^{2+} and histidine was found to be very broad covering the pH from 5.5 to 9.0 well suitable for living cell applications (Fig. S19, ESI).

We also explored the potential applications of the sensing ensemble in biological system. The typical fluorescent images of U87MG cells which have been separately incubated with **CAQA** and **CAQA**-Cu^{2+} at 5 and 10 μM for 12 h, are shown in Fig. 4a-d. According to the visual assessment of these images with these two probes incubated toward hard-to-transfect glioblastoma U87MG cells,[30] intensive green fluorescence can be observed in the cytoplasm (Fig. 4a-b). This observation reveals that a small amount of **CAQA** can be internalized into the U87MG cells. On the other hand, according to the visual assessment of fluorescent images with **CAQA**-Cu^{2+} incubated toward U87MG cells, significant enhancement of fluorescent signal can be observed in the cytoplasm in contrast to the bare **CAQA** (Fig. 4c-d). Somewhat interestingly, the cellular uptake efficiency of the ensemble is enhanced thereby reacting preferentially with cysteine and histidine in the cytoplasm to turn on the fluorescent signal of the device. Conceivably, the fast cellular uptake efficiency of the probe depends on the surface charge density and stability of the composites during the receptor-mediated endocytosis[30] and the subsequent seizure of Cu^{2+} from the ensemble by the biomolecules localized in cytoplasm, leading to the recovery of the fluorescence of **CAQA**.

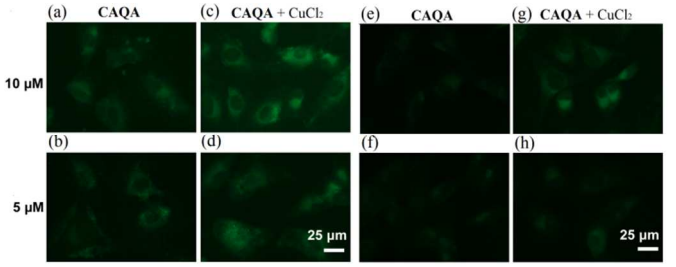


Fig. 4 (a-d) Cellular uptake of the **CAQA** without (a-b) and with (c-d) Cu^{2+} ion at 5 or 10 μM towards Human glioblastoma cells U87MG. (e-h) Cellular uptake of the **CAQA** without (e-f) and with (g-h) Cu^{2+} ion at 5 or 10 μM towards Human glioblastoma cells U87MG after a pre-treatment with NEM (20 μM) and lipofectamine 2000 (4 μL/mL).

In another experiments, U87MG cells were pre-treated with an excess of thiol-scavenger NEM with lipofectamine to consume all free thiols present in the cells. Subsequently, **CAQA** or **CAQA**-Cu^{2+} at 5 or 10 μM was separately incubated with the treated cells thereby observing their fluorescent images in Fig. 4e-h. By monitoring the respective emission images with **CAQA** and **CAQA**-Cu^{2+} incubated toward the **CAQA**-Cu^{2+} pretreated cells, the observed fluorescent signals in the cytoplasm are significantly reduced. Apparently, NEM as expected will silence the action of cysteine toward the probe and the ensemble. Noticeably, both **CAQA** and **CAQA**-Cu^{2+} can be efficiently internalized into hard-to-transfect U87MG cells without the use of any transfecting agents. Secondly, the observable fluorescent signals with **CAQA**-Cu^{2+} toward the treated cells are turned on mainly by the action of the ensemble with histidine moieties within the cytoplasm at the concentration level around 0.1 mM,[31] rendering it an *in vitro* histidine sensor.

The cytotoxicity of both **CAQA** and **CAQA**-Cu^{2+} towards U87MG cells were evaluated by MTT assay (Fig. S20, ESI).[32] Generally, percentage cell viabilities exceeds 95%. These results reveal that both the probe and the ensemble are non-cytotoxic toward U87MG cells.

In summary, we synthesized a novel fluorescent sensing probe **CAQA** for selective detection of Cu^{2+} with low LOD. Coupled the use of *N*-ethylmaleimide, a 1:1 mixture of **CAQA** and Cu^{2+} constituted a sensing ensemble amenable to intracellular

detection of histidine. This ensemble device would be further developed as probes for histidine-related signal transduction in brain and other cancers.

Notes and references

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Electronic Supplementary Information (ESI) available: Experimental sections, NMR spectra, UV-vis spectra, fluorescence spectra and MTT assay. See DOI: 10.1039/c000000x/

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