Fluorescence turn-on detection of Cu^{2+} in water samples and living cells based on the unprecedented copper-mediated dihydrorosamine oxidation reaction†

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A fluorescence turn-on probe for Cu^{2+} based on the novel copper-mediated dihydrorosamine oxidation reaction has been constructed and employed in the detection of Cu^{2+} in water, new born calf serum, and living cells, and the new copper-mediated dihydrorosamine oxidation reaction likely proceeds by a copper redox mechanism.

Spectacular successes have been achieved in the organic synthesis field in the preparation of a wide variety of species. However, the central themes in organic synthesis are carbon–carbon bond formation and functional group transformation. In most cases, functional group transformation may lead to pronounced changes in electronic properties. Thus, if a functional group transformation occurs in close proximity to a fluorescent dye, this will have a significant effect on the photophysical states of the dye resulting in a visible variation in the dye emission profile. This constitutes the basis of the organic reaction-based fluorescent probe development approach.[1]

Oxidation reactions are an important class of organic reactions. Transition metal ions are known to be able to facilitate the oxidation reactions. Thus, transition metal-based oxidations may be exploited to design fluorescent probes for transition metal ions of interest. Recently, the groups of Burdette,[2a] Gopidas,[2b] and Brückner[2c] have elegantly constructed fluorescent probes by metal-based oxidations. However, these probes have not been utilized in sensing metal ions in biological samples likely due to limited biocompatibility. To examine the feasibility of the construction of fluorescent probes based on the metal-mediated oxidation strategy that can function under biocompatible conditions, we were interested in creating Cu^{2+} fluorescent probes by exploiting the oxidation ability of copper, as Cu^{2+} plays a critical role in living systems.[3] To realize this goal, the major challenges include: (1) selection of the oxidation reactions mediated by Cu^{2+} which can proceed readily in biocompatible conditions; (2) transducing the changes in the electronic properties of the probes in light of the oxidation to a marked fluorescence turn-on signal; (3) the stability of the probes in the open air; (4) choice of the oxidation reactions mediated by Cu^{2+} which are clean and complete.

The rosamine dyes have excellent photophysical properties, such as long wavelength absorption and emission, large extinction coefficients, and high fluorescence quantum yields.[4] In the reduced form, the xanthene rings of dihydrorosamines are deconjugated and thus non-fluorescent. Nevertheless, upon oxidation, the xanthene rings transform into their conjugated forms, and the fluorescence is switched on. Some fluorescent probes for reactive oxygen species have been constructed by the oxidation of dihydrorosamines, and they tend to have poor stability due to auto-oxidation in air.[5] However, to the best of our knowledge, dihydrorosamine oxidation by metal ions has been previously unknown, and thus the metal-mediated dihydrorosamine oxidation has not been exploited in the design of fluorescent probes for metal ions. Herein, we present dihydrorosamine **1** (Fig. 1), a new type of fluorescence turn-on copper probe, as the first metal-oxidation based fluorescence turn-on probe that can operate in biological conditions. In addition, we also proposed that the unprecedented copper-mediated dihydrorosamine oxidation reaction employed for the probe development likely proceeds by a copper redox mechanism.

The synthesis of probe **1** is outlined in Scheme S1.† The structure of compound **1** was fully characterized by ^{1}H NMR, ^{13}C NMR, HRMS, and single crystal X-ray diffraction analysis.‡ The single-crystal X-ray crystallographic analysis revealed that compound **1** adopts a deconjugated form and that carbon 9 (labeled as C7 in the ORTEP drawing in Fig. 2) is in the sp^3 hybrid state. The fact that the crystal structure of compound **1** was successfully acquired indicates that it is relatively stable. Notably, this is the first report of a dihydrorosamine crystal structure.

Probe **1** exhibited essentially no emission (Fig. 3) due to its deconjugated structure. However, when probe **1** was titrated

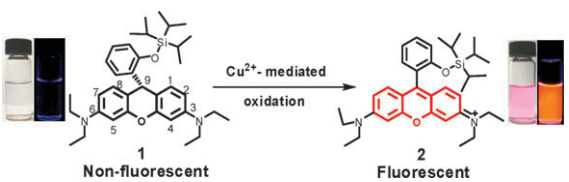

Fig. 1 Structures of probe **1** and its conjugated compound **2**; visible color and visual fluorescence color of probe **1** and compound **2** on excitation at 365 nm using a handheld UV lamp. The oxidation reaction proceeded in 20 mM HEPES buffer, at pH 7.4, containing 40% CH_3CN as a cosolvent.

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Fig. 2 ORTEP diagram of compound **1**. Notably, carbon 9 is labeled as C7.

with increasing concentrations of Cu^{2+}, a drastic increase in the featured emission of conjugated rosamine around 580 nm was observed, and the fluorescence intensity enhancement was up to 100-fold. Consistently, the visual emission color of the probe **1** solution turned from dark to bright orange (Fig. 1). Moreover, the probe showed high sensitivity toward Cu^{2+} with a detection limit of 2.61×10^{-7} M (S/N = 3), which is superior or comparable to most of the reported fluorescent Cu^{2+} probes[2b,6,7] and is much lower than the typical concentration of blood copper (11.8–23.6 μM) in normal individuals[8] and the limit of copper in drinking water (~20 μM) set by the U.S. Environmental Protection Agency.

We then proceeded to examine the selectivity of the probe. As shown in Fig. 4, introduction of Cu^{2+} to the probe **1** solution resulted in a significant enhancement in the fluorescent intensity at 580 nm. By contrast, no marked changes in the emission were noted upon addition of the representative species such as Ag^{+}, Al^{3+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Fe^{2+}, Fe^{3+}, Hg^{2+}, Mn^{2+}, Ni^{2+}, Pb^{2+}, Zn^{2+}, F^{-}, NO_2^{-}, H_2O_2 + horseradish peroxidase (HRP), and $^{\bullet}OH$, indicating that the probe has a high selectivity for Cu^{2+}. This is also supported by the observation that other species have negligible interference with the fluorescence response. The absorption spectra also showed the similar selectivity to the species (Fig. S2†). Furthermore, the visual response of probe **1** to various species (Fig. S3†) demonstrates that the probe can be used conveniently for Cu^{2+} detection by simple visual inspection.

Probe **1** could be employed to detect Cu^{2+} in the pH range 3.0–8.0 (Fig. S4†). This indicates that the probe functions properly at physiological pH. The solid powder of probe **1** was stable at −20 °C for at least one year, and there were no obvious changes in the fluorescence intensity of probe **1** in the assay solution for three days at room temperature (Fig. S5†).

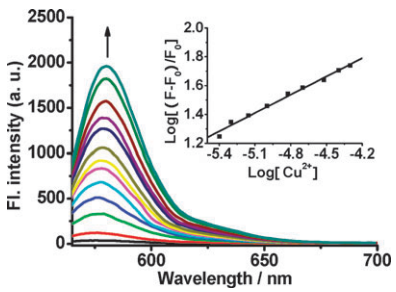

Fig. 3 Fluorescence spectral changes of probe **1** (10 μM) upon addition of increasing concentrations (0–5 equiv.) of Cu^{2+} (λ_{ex} = 554 nm) in 20 mM HEPES buffer, pH 7.4, containing 40% CH_3CN as a cosolvent. The inset shows the correlation between the fluorescence intensity and copper concentration.

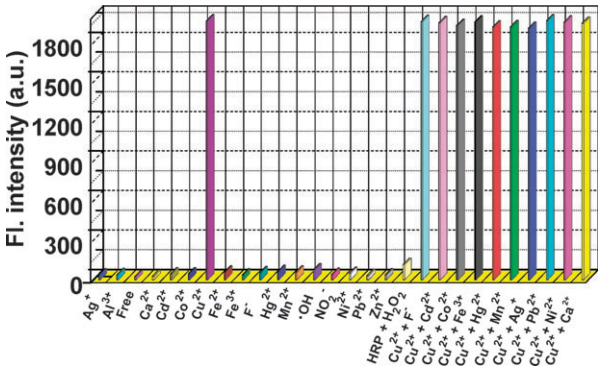

Fig. 4 The fluorescence intensity of probe **1** (10 μM) at 580 nm in the absence and presence of different species (5 equiv.) in 20 mM HEPES buffer, pH 7.4, containing 40% CH_3CN as a cosolvent.

Thus, probe **1** has much less auto-oxidation and is significantly more stable than other dihydrorosamine-based reactive oxygen probes.[5] Presumably, the bulky isopropyl silane group may contribute to the stability.

The conjugated product of probe **1** + Cu^{2+} was isolated by column chromatography, and its identity was confirmed to be conjugated rosamine **2** (fluorescence quantum yield Φ_f = 0.22) based on the ^1H NMR, ^{13}C NMR, mass spectrometry, absorption, emission, and excitation spectroscopic studies (Fig. S6–S11†). The oxidation conversion of probe **1** to compound **2** essentially reached 100% in the presence of 5 equiv. Cu^{2+} at 30 min (Fig. S12†), indicating that the oxidation reaction is clean and complete. This is highly desirable for reaction-based probes, as the fluorimetric assay should be much simpler and more reliable. As the oxidation reaction was essentially complete in 30 min (Fig. S13†), in this work, an assay time of 30 min was selected in the evaluation of the sensitivity and selectivity of probe **1** toward Cu^{2+}.

Although copper ions are known to facilitate the oxidation of phenolic compounds,[9] the Cu^{2+}-mediated oxidation of dihydrorosamine has not been revealed in the literature and the oxidation mechanism is unknown. To gain insight into the likely mechanism of the novel Cu^{2+}-mediated oxidation of dihydrorosamine, we investigated the kinetic profile of the formation of conjugated compound **2** from probe **1** in the presence of different equiv. of Cu^{2+} by absorption spectroscopy. When probe **1** (10 μM) was treated with various equiv. of copper ions, the maximal absorption at 558 nm could be reached after 30 min and 215 h in the presence of 5 and 0.1 equiv. Cu^{2+}, respectively (Fig. 5). This preliminary kinetic study implies that the Cu^{2+}-mediated oxidation plays an important role in the oxidation of non-fluorescent probe **1** to fluorescent rosamine **2**.[9] The presence of Cu^{+} in the process was detected by the Cu^{+} indicator neocuproine in light of the formation of an absorption band around 460 nm (Fig. S14†), attributed to the $[Cu(neocuproine)_2]^{+}$ complex.[10] These findings suggest that the oxidation process likely involves a copper redox mechanism.[9]

The linear calibration curve (the inset in Fig. 3) indicates that the probe may be employed to determine copper concentration in water. Indeed, probe **1** was able to quantitatively detect various concentrations of Cu^{2+} spiked in water samples from Yuelu spring with excellent recovery (Table S1†). Thus,

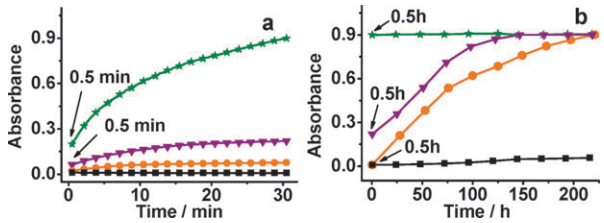

Fig. 5 The variations of absorbance of probe **1** (10 μM) in 20 mM HEPES buffer, pH 7.4, containing 40% CH_3CN as a cosolvent in the presence of 0 equiv. (■), 0.1 equiv. (●), 0.5 equiv. (▼) and 5 equiv. (★) Cu^{2+} as a function of time. (a) The variations of the absorbance in the initial 30 min (notably, it took roughly 0.5 min to mix the solution and record the spectra); (b) the variations of the absorbance from the subsequent 30 min to 215 h. The absorbance was recorded around 558 nm at room temperature.

probe **1** is satisfactory for monitoring copper in environmental water samples. Furthermore, probe **1** could also sense Cu^{2+} in new born calf serum by simple visual inspection (Fig. S15†).

The utility of probe **1** for fluorescence imaging of Cu^{2+} in living cells was investigated. Staining of nasopharyngeal carcinoma cells with only probe **1** provided no significant fluorescence (Fig. 6a). In Fig. 6b, the cells were pre-treated with Cu^{2+} in the growth medium for 30 min. The cells were then washed with PBS to remove the remaining Cu^{2+} and further incubated with probe **1** for 30 min. The resulting bright fluorescence image demonstrates that probe **1** with suitable amphipathicity is cell membrane permeable and able to display a fluorescence turn-on response to Cu^{2+} in the living cells.

The desirable features of probe **1** include high sensitivity and selectivity for Cu^{2+}, working well in biocompatible conditions, the clean and complete oxidation reaction mediated by Cu^{2+}, and satisfactory stability in the open air. Notably, one striking character of probe **1** is a fluorescence turn-on response by circumventing the intrinsic fluorescence quenching nature of paramagnetic Cu^{2+}. This may be considered as the advantage of the metal-oxidation based probe development strategy.

In conclusion, we have created a novel type of fluorescent copper probe **1** based on the new copper-mediated dihydrorosamine oxidation reaction, and the probe has been employed to sense Cu^{2+} in water, new born calf serum, and living cells. Notably, probe **1** represents the first fluorescence turn-on probe on the basis of metal-mediated oxidation that can work in biological conditions. Furthermore, we also proposed that the novel copper-mediated dihydrorosamine oxidation reaction likely proceeds by a copper redox mechanism. We expect that the general metal-mediated oxidation approach could be broadly extended to develop biocompatible fluorescent probes for various metal ions of interest. Our research is now in progress along this line.

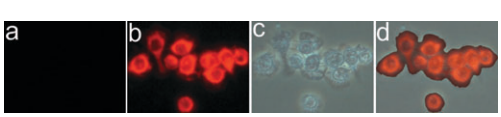

Fig. 6 Fluorescence and brightfield images of cells. (a) Cells stained with probe **1** (10 μM) for 30 min at 37 °C; (b) cells pre-treated with Cu^{2+} (5 equiv.) for 30 min at 37 °C and then further incubated with probe **1** (10 μM) for 30 min at 37 °C; (c) brightfield image of cells shown in pane b; (d) an overlay image of (b) and (c). The cell culture and imaging conditions are in accordance with those in ref. 6*a*,7*a*,*b*,*g*.

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Notes and references

‡ Crystal data for probe **1**: $C_{36}H_{52}N_2O_2Si$, M = 572.89, monoclinic, space group $P2_1/c$, a = 19.8209(19), b = 13.1032(12), c = 13.9853(13) Å, α = 90.00, β = 108.316(2), γ = 90.00°, V = 3448.2(6) Å^3, T = 293(2) K, Z = 4, D_c = 1.104 Mg m^{-3}, F_{000} = 1248, μ(MoKα) = 0.100 mm^{-1}, λ = 0.71073 Å, $2\theta_{max}$ = 51.0°. 17917 reflections measured, 6406 unique (R_{int} = 0.1102). The structures were solved by direct methods and refined by a full-matrix least-squares technique on F^2 using the SHELXL97 program. Final GooF = 0.859, R_1 = 0.0769, wR_2 = 0.1948, R indices based on 2743 reflections and 375 refined parameters, with $I > 2\sigma(I)$. CCDC 748393.

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