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A FRET-based rhodamine–benzimidazole conjugate as a Cu^{2+}-selective colorimetric and ratiometric fluorescence probe that functions as a cytoplasm marker†

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On the basis of fluorescence resonance energy transfer (FRET) from benzimidazole to a rhodamine moiety, a rhodamine–benzimidazole conjugate (RBC) ratiometric fluorescent probe has been designed and synthesized. The RBC selectively binds to Cu^{2+}, showing visually observable changes in absorption and emission behavior, and demonstrates an effective intracellular Cu^{2+} imaging ability, allowing it to function as a cytoplasm marker.

Introduction

Fluorescent chemosensors which display a significant change in optical signal when they selectively sense a specific guest analyte, especially for metal ions of biological interest, have several advantages over other methods such as their sensitivity, specificity and real-time monitoring with fast response times.[1,2] However, variation in the fluorescence intensity without much shift in either the excitation or emission wavelengths, can be influenced by many environmental aspects, the emission collection efficiency, the effective cell thickness in the optical beam and changes in the excitation intensity.[3] To reduce the influence of such factors, ratiometric measurement is utilized, where simultaneous recording of the fluorescence intensities at two wavelengths and computation of their ratio is carried out.[4] This technique provides greater precision than measurement at a single wavelength, and is suitable for cellular imaging studies.

The design of fluorescent probes is generally based on intramolecular charge transfer (ICT), photo-induced electron transfer (PET), chelation-enhanced fluorescence (CHEF), metal–ligand charge transfer (MLCT), excimer/exciplex formation, imine isomerization, intermolecular hydrogen bonding, excited-state intra-molecular proton transfer, and fluorescence resonance energy transfer (FRET).[5–12] FRET, which is our present interest, is defined as an excited-state energy interaction between two fluorophores in which the excited donor energy is transferred non-radiatively to an acceptor unit. Among these mechanisms using a single fluorophore to obtain ratiometric changes, the use of multi-fluorophores with energy donor–acceptor architectures can achieve large pseudo-Stokes shifts. This approach can provide simultaneous recording ratio signals for two emission intensities at different wavelengths, which could afford a built-in correction for environmental effects[13] and supply a facile method for DNA detection, the labeling of proteins and other biomarkers, and for visualizing complex biological processes at the molecular level, such as nucleic acid regulation.[14] Thus, FRET is one of the most commonly used chemical principles for ratiometric imaging, whether *in vitro* or *in vivo*.

Copper(II) plays a significant role in various fields.[15,16] However, exposure to high concentration levels of copper, even for a short period of time, can cause gastrointestinal disturbances, while long-term exposure can cause liver or kidney damage.[17–20] Furthermore, Cu^{2+} can act as a significant environmental pollutant because of its extensive use in industry and agriculture. Thus, the fast detection of Cu^{2+} in environmental and biological samples has become increasingly critical, not only due to its important role in biological processes, but also, because of its high toxicity to organisms at increased concentration levels.[21]

Considerable efforts have been made to synthesize fluorescent chemosensors that are selective, sensitive and suited to high-resolution imaging for monitoring biological processes.[22] Even though great achievements in the field of colorimetric and/or fluorescent chemosensors for Cu^{2+} have been reported,[23–31] there is still a demand to develop new indicators with improved properties. Due to the significant physiological relevance and associated biomedical implications, the design and

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development of selective and sensitive sensors directed toward the detection and measurement of divalent Cu^{2+}, the third most abundant essential trace element after iron and zinc in the human body, is highly desirable. There are only a few reports on FRET-based ion chemosensors.[32,33] In particular, the design of ratiometric probes for Cu^{2+} ions is a challenge due to their inherent paramagnetic nature, and hence, complexation generally results in quenching of the fluorescence intensity of the probe.

Among the fluorophores developed, rhodamine is highly favored because of its photophysical properties, which include a high extinction coefficient, a high quantum yield and remarkable photostability. In general, spirolactam ring opening of a rhodamine derivative on complexation with a metal ion gives rise to a color change and strong fluorescence.[34–39]

Motivated by the biological importance of Cu^{2+}, herein we report the ratiometric metal ion sensing capability of a benzimidazole–rhodamine conjugate (RBC) and its absorption and emission activities upon metal complexation for detecting Cu^{2+} and its effective bioimaging. In the present study, our design strategy for the detection of Cu^{2+} is based on modulating the FRET process in a fluorophore dyad comprising the rhodamine acceptor and the benzimidazole derivative as a donor, linked by a multi-chelating site.

Results and discussion

The fluorescent RBC probe was obtained from the reaction of rhodamine B hydrazide[40] (**1**) with 2-chloromethyl-1-methyl-1*H*-benzimidazole (**2**) in refluxing THF for two days as shown in Scheme 1. The molecular structure of the RBC was confirmed using ^1H NMR and ^{13}C NMR spectroscopy, and HRMS (ESI).

As the RBC bears two different fluorophore units, we considered it appropriate to study the metal binding event at two different excitation wavelengths corresponding to the benzimidazole unit (315 nm) and the xanthene unit (495 nm). In the absence of Cu^{2+}, the rhodamine moiety adopts a closed, non-fluorescent spirolactam form, corresponding to a weak spectral overlap between benzimidazole emission and rhodamine absorption.

The binding of Cu^{2+} induces opening of the spirolactam ring in the RBC with an associated switch to a UV-vis spectral response in the 500–580 nm range, which has a significant spectral overlap with the emission spectrum of the benzimidazole fragment (shaded black area in Fig. 1), which could provide a plausible route for the non-radiative transfer of excitation energy from the donor benzimidazole to the acceptor rhodamine moiety, thereby initiating an intramolecular FRET process.

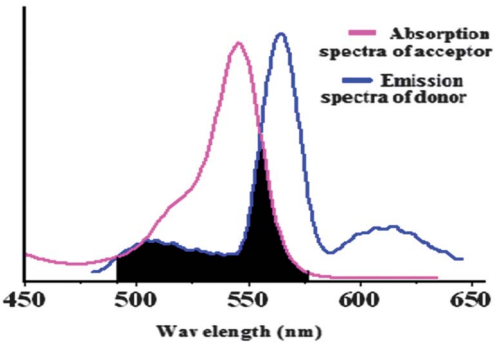


Fig. 1 Spectral overlap between benzimidazolyl (10 μM) emission (blue line) and ring-opened rhodamine B (10 μM) absorption (pink line).

The photophysical properties of the RBC were investigated in an acetonitrile–HEPES buffer solution (1 mM, pH 7.4; 8 : 2 v/v). As expected, the RBC barely displayed absorbance at 450–650 nm, indicating that the RBC exists in a spiro-cycle closed form, and instead, shows an absorption band centered at 315 nm. The gradual addition of Cu^{2+} to a solution of the RBC caused a significant enhancement in absorbance (Fig. 2) located at 556 nm, while the absorbance at 315 nm diminished slowly.

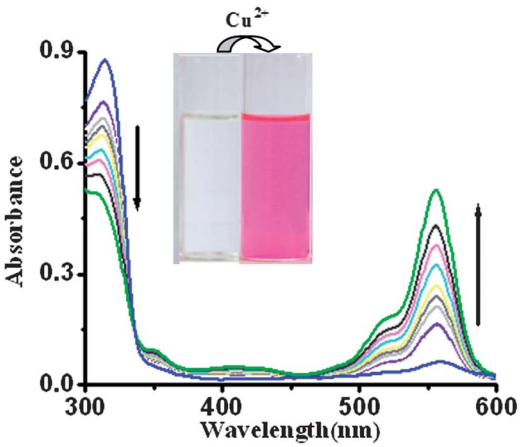
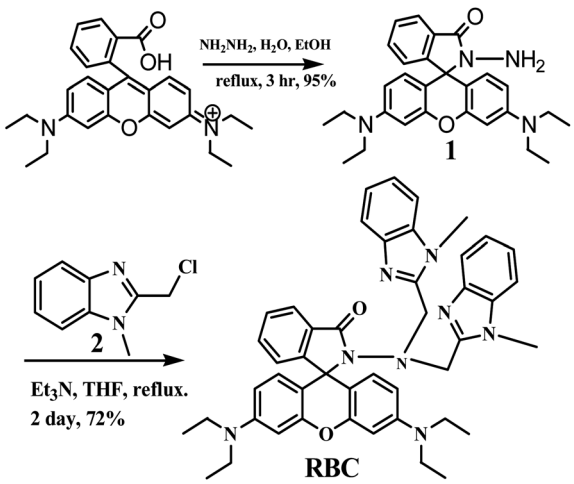


Fig. 2 UV-vis titration spectra of the RBC (10 μM) with varying [Cu^{2+}] from 0 to 3 equiv in a CH_3CN–HEPES buffer solution (1 mM, pH 7.4; 8 : 2 v/v).

These results indicate that chelation with Cu^{2+} induced the development of a pink color as a result of ring opening of the spirolactam form of the RBC.



Scheme 1 Synthesis of the RBC.

In the presence of competing metal ions such as Mn^{2+},Cr^{3+}, Pb^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Cd^{2+} and Hg^{2+} (as perchlorate, nitrate or chloride salts), in turn, no significant absorbance (Fig. 3) was observed at 556 nm that could interfere with the selectivity of the RBC for the detection of Cu^{2+}. These results show that the RBC is a Cu^{2+}-specific probe that allows the visual detection of Cu^{2+}.

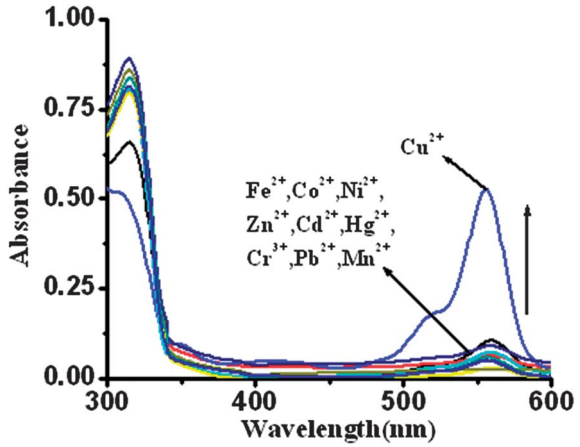


Fig. 3 Changes in the absorption spectra of the RBC–Cu^{2+} complex in the presence of different metal ions.

The selectivity of the RBC towards Cu^{2+} binding was also observed through fluorescence titration. The RBC was excited at 315 nm, which is the excitation wavelength of the benzimidazole moiety. In the absence of Cu^{2+}, the fluorescence emission peak appeared at 490 nm. Upon the gradual addition of Cu^{2+}, a significant decrease in the emission intensity at 490 nm and a new fluorescence emission band centered at 580 nm were observed, with a clear iso-emissive point at 553 nm (Fig. 4), which resulted in an intense reddish orange color.

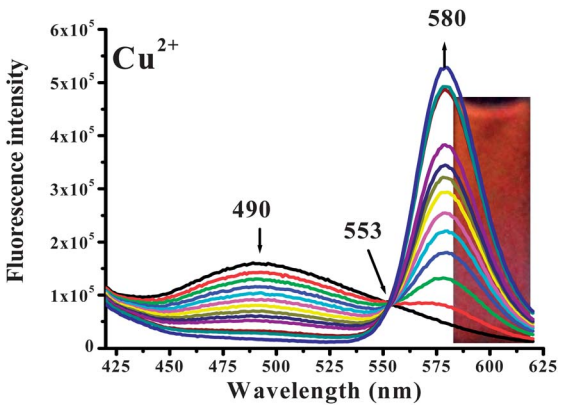
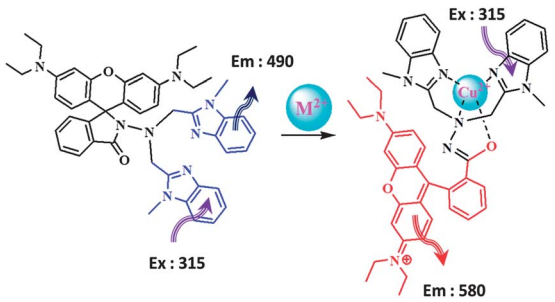


Fig. 4 Fluorescence titration spectra (λ_{ex} = 315 nm) of the RBC (10 μM) upon the incremental addition of 0 to 3 equiv of Cu^{2+}, in an CH_3CN–HEPES buffer solution (1 mM, pH 7.4; 8 : 2 v/v).

This is due to the fact that binding of Cu^{2+} with the RBC induces spirolactam ring opening of the rhodamine derivative, whose absorption spectrum shows a significant spectral overlap



Scheme 2 Cu^{2+}-induced FRET OFF → ON of the RBC.

with the emission spectrum of the benzimidazole moiety. This facilitates the resonance energy transfer process (Scheme 2), and hence, a pseudo-large Stokes shift based on the FRET mechanism is observed . In the presence of Cu^{2+}, the ratio of the emission intensities for rhodamine to the benzimidazole moiety at 580 nm and 490 nm (I_{580}/I_{490}) increases substantially from 0.28 in the absence of Cu^{2+} to 15.1 in the presence of Cu^{2+} (up to 3 equiv.).

A competition experiment was also performed by adding Cu^{2+} (3.0 equiv.) to a RBC solution in the presence of commonly employed interfering metal ions (3.0 equiv.). The selectivity profile diagram (Fig. 6) reveals that Cu^{2+}-induced fluorescence enhancement (I_{580}/I_{490}) remains unaffected by the co-existence of other metal ions, and interference does not occur.

Thus, the RBC exhibits excellent selectivity for Cu^{2+} and is insensitive to interference from other metal ions (Fig. 5). Only Fe^{2+} and Co^{2+} showed some emission at around 580, but they could not compete with Cu^{2+}. Fluorescence titration and Job plot analysis (Fig. S1†) confirmed a 1 : 1 stoichiometry for Cu^{2+} and the RBC, with an association constant of 1.5 × 10^4 M^{-1} (Fig. S_2†) and a detection limit of 3.1 μM (Fig. S3†).

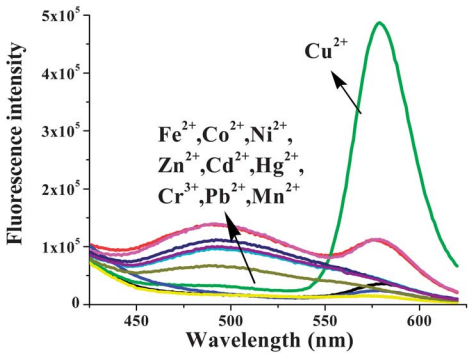


Fig. 5 Changes in the fluorescence emission of the RBC (10 μM) observed upon addition of different metal ions in a CH_3CN–HEPES buffer solution (1 mM, pH 7.4; 8 : 2 v/v).

The mass spectrum (ESI) of the RBC–Cu^{2+} complex (expected M^+) shows a molecular-ion peak at m/z 425.27 $[C_{48}H_{51}CuN_9O_2 + 2H]^{2+}$ = 425 corresponding to [RBC + Cu + $CH_3CN]^{2+}$(ESI) $[C_{48}H_{51}CuN_9O_2 + 2H]^{2+}$ = 425, which suggests a probable mode of binding, as proposed in Fig. 7.

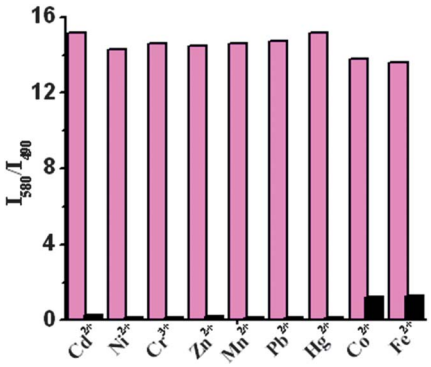


Fig. 6 Metal ion selectivity profile of the RBC sensor (10 μM): change in emission intensity of the RBC + 3.0 equiv. M^{n+} (black bars); change in emission intensity of the RBC + 3.0 equiv. M^{n+}, followed by 3.0 equiv. Cu^{2+} (pink bars) at I_{580}/I_{490}.

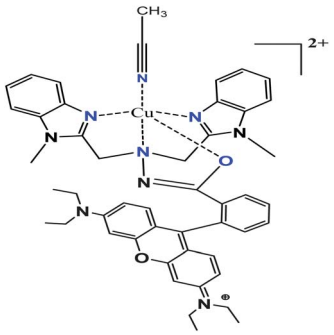


Fig. 7 Probable mode of binding of the RBC–Cu^{2+} complex in acetonitrile.

Cell imaging

Due to the favorable binding properties of the RBC with respect to Cu^{2+}, and its intense emission in the visible region, it was conceived that it could be exploited for practical bioimaging, particularly for the sensitive detection of intracellular Cu^{2+}. In order to determine the membrane permeability of the RBC receptor and its ability to specifically bind Cu^{2+} ions in living cells, HeLa cells were first incubated with $CuCl_2$ followed by the addition of the RBC. A control experiment, *i.e.*, incubation with the RBC only, was also carried out. As shown in Fig. 8, the cytoplasm of the cells showed intense red fluorescence in the red channel when they were treated with $CuCl_2$ followed by the RBC, but no fluorescence was observed in cells that were treated with the RBC only in the same channel. As expected, a strong blue fluorescence could be obtained from the nucleus due to DAPI treatment.

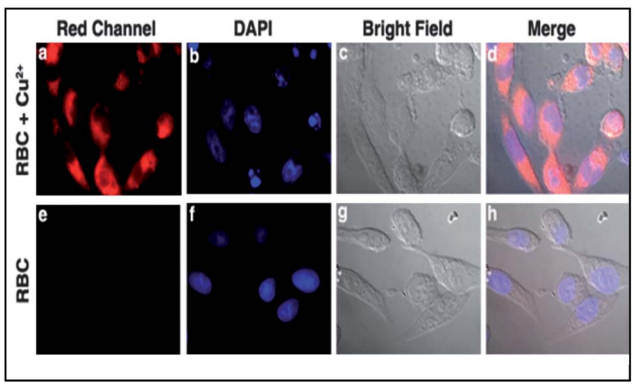


Fig. 8 Fluorescence images of HeLa cells incubated with 50 μM of the RBC in the presence (a and b) and in the absence (e and f) of 50 μM of $CuCl_2$. The corresponding bright field images (c and g) and merge images (d and h) of the cells are shown.

The results clearly indicate that the RBC receptor not only permeates the plasma membrane of the cells, but also brings about a specific cytoplasmic fluorescence in the presence of Cu^{2+} ions. Therefore, the RBC receptor could act as a good cytoplasmic marker in the presence of Cu^{2+} ions.

Conclusions

In summary, we have developed a sensitive RBC probe which selectively binds to Cu^{2+} ions and induces a switch ON response in fluorescence spectra in the visible region. In addition to this, the FRET-based fluorescence response makes it a dual probe for naked eye detection through changes in color and fluorescence. The detection limit for Cu^{2+} was found to be much lower than the standard permissible Cu^{2+} concentrations in drinking water. Furthermore, the RBC demonstrates good bioimaging ability as a cytoplasmic marker in the presence of intracellular Cu^{2+}.

Experimental section

General

Unless otherwise mentioned, chemicals and solvents were purchased from Sigma-Aldrich, and were used without further purification. ^{1}H-NMR spectra were recorded on a Bruker 400 MHz instrument. For NMR spectra, $CDCl_3$ was used as a solvent with TMS as an internal standard. Chemical shifts are expressed in δ units and as 1H–1H and 1H–C coupling constants in Hz. UV-vis titration experiments were performed on a JASCO UV-V530 spectrophotometer and fluorescence experimentation was carried out using a PerkinElmer LS 55 fluorescence spectrophotometer using a fluorescence cell of 10 mm path.

General method for UV-vis and fluorescence titrations

For UV-vis and fluorescence titrations, a stock solution of the RBC was prepared ($c = 1 \times 10^{-5}$ ML^{-1}) in an acetonitrile–HEPES buffer solution (1 mM, pH 7.4; 8 : 2 v/v). Solutions of the guest metal ions, including Cr^{3+}, Ni^{2+}, Cu^{2+}, Pb^{2+}, Co^{2+}, Mn^{2+}, Cd^{2+}, Fe^{2+}, Zn^{2+}, and Hg^{2+} (chloride salts), were prepared ($c = 2 \times 10^{-4}$ ML^{-1}) in CH_3CN. The original volume of the RBC solution was 2 ml. RBC solutions of various concentrations, and increasing concentrations of the metal ions, were prepared separately. The absorption and fluorescence sensing of the metal ions were then recorded.

Cell culture and imaging

HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and penicillin–streptomycin (0.5 U/ml of penicillin and 0.5 μg ml^{-1} streptomycin), on a cover slip in 35 mm dishes at 37 °C, in an atmosphere of air with 5% CO_2 and constant humidity. The cells were initially incubated with the addition of 50 μM of $CuCl_2$ in the growth medium for 30 minutes. After washing three times with phosphate-buffered saline (PBS), fresh growth medium containing 50 μM of the of RBC was added and the cells were incubated for another 30 minutes. Following incubation, the cells were washed three times with PBS and cross-linked with 4% HCHO. Finally, the cover slip was placed on a glass slide containing a DAPI solution and imaging was carried out using a Zeiss Axio Observer Fluorescence Microscope equipped with an ApoTome apparatus.

Synthesis of the RBC

2-Amino-3′,6′-bis(diethylamino)spiro[isoindoline-1,9′-xanthen]-3-one (**1**) (500 mg, 1.1 mmol) and 2-chloromethyl-1-methyl-1*H*-benzimidazole (**2**) were refluxed in dry THF in the presence of Et_3N for two days. The mixture was extracted with chloroform. The combined organic layer was dried over anhydrous magnesium sulfate and then filtered. The filtrate was concentrated and purified through column chromatography using 10% CH_3OH in $CHCl_3$, to give a 72% yield.

^1H NMR ($CDCl_3$, 400 MHz): δ (ppm): 7.93 (s, 2H), 7.41 (s, 4H), 7.08 (s, 2H), 6.45 (d, 7H, $J = 8.00$ Hz), 6.28 (d, 3H, $J = 8.00$ Hz), 3.62 (s, 4H), 3.32 (s, 14H), 1.16 (s, 12H).

^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm): 166.05, 153.84, 151.58, 148.84, 132.48, 130.03, 128.04, 123.50, 122.90, 108.01, 104.63, 98.00, 65.86, 44.35, 12.62.

HRMS ($M + H^+$): calcd 745.2978, found 745.2430. Anal calcd for $C_{46}H_{48}N_8O_2$: 74.17% C, 6.49% H, 15.05% N, 4.30% O; found: 74.26% C, 6.90% H, 15.18% N, 4.68% O.

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