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A lysosomal-targeted fluorescent probe for detecting Cu^{2+}

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ABSTRACT

Copper in lysosomes is closely related to age and development of liver damage. A new fluorescent lysosomes-specific Cu^{2+}-sensing probe was synthesized, and used for optical imaging in HL-7702 and HepG2 cells. Based on the difference in structure between spirocyclic (non-fluorescent) and open-cycle (fluorescent) forms, the probes showed an 80-fold fluorescence increase upon the addition of Cu^{2+}. The probe showed excellent selectivity toward copper ion over other metal ions. A good linear correlation was obtained between fluorescent intensity and the concentrations of Cu^{2+} in the range from 1.0×10^{-7} to 5.5×10^{-6} M, with the detection limit as low as 80 nM. In particular, without appended targeting moieties, it can efficiently accumulate in lysosomes of normal liver and hepatoma cell lines, where it can specifically respond to Cu^{2+} level fluctuations in living cells, including adscititious and endogenous Cu^{2+} level decrease upon ascorbate-reduction, and it displayed good membrane-permeability and low cytotoxicity.

1. Introduction

Lysosomes are cellular organelles that contain acid hydrolase enzymes to break down waste materials and cellular debris. Lysosomes are the terminal degradative compartment of mammalian cells but are also involved in antigen processing, bone remodeling, the regulation of growth factors, and plasma membrane repair [1–4]. In this context, an amount of copper accumulates in lysosomes with increasing age and development of liver damage. This copper initiates lysosomal lipid peroxidation, leading to hepatocyte necrosis [5]. So, hepatoxicity during the development of liver injury is closely related to the level of copper in lysosomes. Therefore, it is appealing to develop small-molecule fluorescent probes sensing relevant biological functional components including Cu^{2+} in lysosomes [6–10]. In the past few years, Kristine Glunde's group has synthesized a novel probe for fluorescence imaging of lysosomes in breast tumors, and Sarah Fakih's group has reported a fluorescent probe to monitor lysosomal iron [11]. However, ideal fluorescent probes that can successfully detect Cu^{2+} in the lysosomes of living cells are rare. Hence, it is desired to devise new suitable probes targeted to lysosomes for imaging concentration fluctuation of labile Cu^{2+}.

To develop a ideal fluorescence probe directing to lysosomes, we selected rhodamine B as the lysosomal-targeted fluorophore because of the N,N-diethylaminophenyl matching pK_a value of lysosomes [12]. And 2-acetyl-thiophene was chosen as a Cu^{2+}-binding unit. Taken together, we designed and synthesized a new fluorescent probe, RHAT (Scheme 1), to detect Cu^{2+}. Without Cu^{2+}, RHAT is a spirocyclic form, colorless and non-fluorescent. While, addition of Cu^{2+} causes the spirocycle opening and RHAT shows concomitantly a significant fluorescence enhancement and an appearance of pink color.

2. Experimental

2.1. Reagents and apparatus

Rhodamine B and TPEN were purchased from Sigma–Aldrich Co. Lyso-Tracker DND-26 was from molecular probes company. All other reagents and solvents were purchased from commercial sources and of analytical reagent grade, unless indicated otherwise. HL-7702 and HepG2 cells were purchased from American Type Culture Collection, Manassas, VA, USA. Silica gel (200–300 mesh, Qingdao Haiyang Chemical Co.) was used for column chromatography. Analytical thin-layer chromatography was performed using GF254 silica gel (precoated sheets, 0.77 mm thick, Taizhou Si-Jia Biochemical Plastic Company). Ultrapure water (18.2 MΩ cm, Sartorious, Germany) was used throughout. Fluorescence measurements were performed on a FS920 fluorescence spectrophotometer FL920 (Edinburgh Instruments, Livingston, UK) using a Xenon lamp and 1 cm^2 quartz cuvettes. All pH measurements were taken with a pH-3c digital pH-meter (Shanghai Lei Ci Device Works, China) with a combined glass-calomel electrode. ^1H NMR, ^{13}C NMR spectra were taken on a Bruker 300 MHz spectrometer (American).

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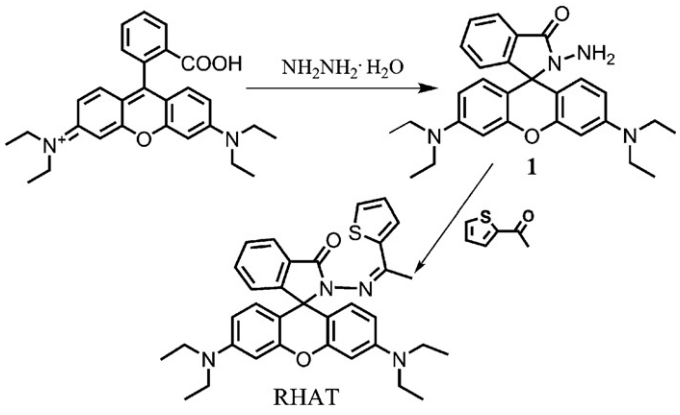

Scheme 1. Synthesis of RHAT.

Molecular weight was acquired from UPLC-LTQ orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, CA). The kinetics experiment was obtained from Varian Cary Eclipse spectrofluorometer (Australian) with a Xenon lamp at room temperature.

2.2. Sample preparation

RHAT was dissolved in CH_3CN as a stock solution (0.10 mM). Aqueous metal ions solutions of Zn^{2+}, Hg^{2+}, Co^{2+}, Ni^{2+}, Cd^{2+} and Mn^{2+} were prepared from the acetates. Solutions of Na^+, K^+, Ca^{2+}, Fe^{3+} and Mg^{2+} were prepared from chlorides. The Fe^{2+} solution was prepared immediately before use with ferrous ammonium sulfate.

2.3. Cell culture

Live human normal liver cells (HL-7702) and human liver carcinoma cell line (HepG2) were purchased from Institute of Biochemistry and Cell Biology of Chinese Academic. Cells were grown in RPMI 1640 Medium and incubated at 37 °C in a 5% CO_2/95% air humidified incubator (MCO-15AC, SANYO). Cells were seeded at a density of 1.0×10^6 cells mL^{-1} for confocal imaging supplemented with 10% fetal bovine serum (Gibco, Invitrogen), $NaHCO_3$ (2.0 g/L) and 1% antibiotics (penicillin/streptomycin, 100 U mL^{-1}).

2.4. Confocal microscopy

Cells were passed and plated on 18 mm glass coverslips at 37 °C, 5% CO_2 1 day before imaging. A Leica TCS SP2 laser scanning microscopy system containing a 20× air-immersion objective lens (HCXApo) was used for the probe experiments. Confocal images of liver cells fluorescence of the RHAT were collected using a 543 nm excitation light from a Helium/Neon II – 5 mW laser, the collection window is 560–590 nm. The excitation wavelength of Lyso-Tracker DND-26 was 488 nm, the collection window is 500–530 nm. The medium was removed and cells were washed with HEPES (10 mM, pH 6.85) for three times.

3. Results and discussion

3.1. Spectroscopic properties and optical responses to Cu^{2+}

Initially, the response of RHAT to Cu^{2+} was investigated, and the optimal measurement conditions were established, such as probe concentrations, pH, HEPES buffer aqueous solution concentrations (Supporting information). RHAT shows excitation at 555 nm and emission at 576 nm ($\Phi_F = 0.0097$). The fluorescence rapidly exhibits a significant enhancement (80-fold) with Cu^{2+} presence ($\Phi_F = 0.39$) (Fig. S1).

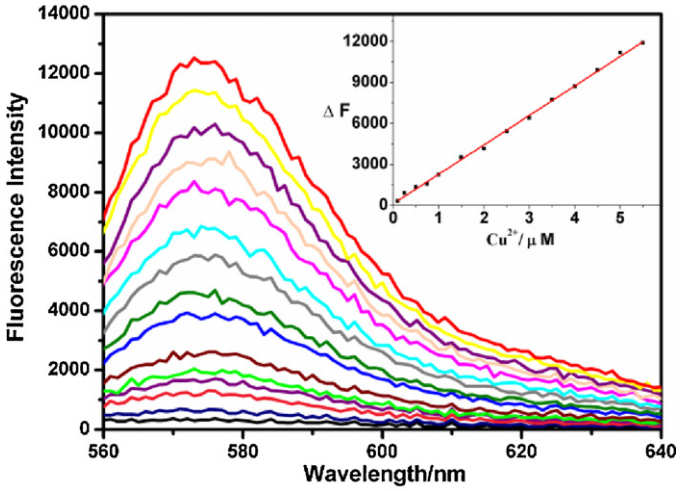

Fig. 1. Fluorescence responses of RHAT (15 μM) toward different concentrations of Cu^{2+} (0, 0.10, 0.25, 0.50, 0.75, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5 μM, from bottom to top) in buffered aqueous solution (10 mM HEPES, pH 6.85). Spectra were obtained with excitation at 555 nm. Inset: plot of relationship fluorescence intensities at 576 nm depending on the concentration of Cu^{2+}.

3.2. The relationship between RHAT and Cu^{2+}

Based on Lambert-Beer law, the molar absorptivity was calculated to 1.63×10^4 L mol^{-1} cm^{-1} and the Sandell sensitivity 3.9 ng cm^{-2} of Cu^{2+} per 0.001 A. The low values of Sandell sensitivity indicated the high sensitivity of the proposed method. An obvious color change from colorless to pink was observed by the naked eyes. Moreover, there was a good linear correlation ($R = 0.9982$) between fluorescent intensity and Cu^{2+} concentration in the range from 1.0×10^{-7} to 5.5×10^{-6} M. The regression equation was $F = 2147.5 + 426.2$ [Cu^{2+}] (μM) (Fig. 1) and the detection limit is calculated to be 80 nM. This result showed that RHAT could measure Cu^{2+} qualitatively and quantitatively.

The Job plot analysis revealed that the inflection point was at ~0.5, indicating that RHAT forms a 1:1 species with Cu^{2+} in solution (Fig. S2). According to the linear Benesi–Hildebrand expression [13,14], the association constant (K_d) of RHAT with Cu^{2+} ion in water was found to be 0.16 μM.

3.3. Effect of pH

By the pH experiment, in the range of 4.5–6.0, we found the fluorescence emission spectrum showed a remarkable enhancement correspondingly in the presence of Cu^{2+}. As far as we all known, lysosomes contain several types of hydrolytic enzymes, which maintain an acidic lumenal pH of ~5.0 [15]. We surmised that RHAT was suitable to detect Cu^{2+} in lysosomes (Fig. S3).

3.4. Selectivity, stability and reversibility

To assess the selectivity of the method, the interference of other metal ions on the determination of Cu^{2+} was examined. As shown in Fig. 2, no distinct responses were observed upon the addition of other metal ions. The interference of co-existent intracellular cations was negligible on Cu^{2+} sensing, even when Na^+, K^+, Mg^{2+} and Ca^{2+} were present at millimolar levels. These results suggested that RHAT was a desirable probe for the fluorescence detection of the Cu^{2+} with high selectivity. To testify the stability of the probe, the kinetic experiments were performed (Fig. S4). The results showed that the probe and complex had superior photostability, showing enough immunity to the medium effect. Additionally, during the experiment, the experimental results are consistent though

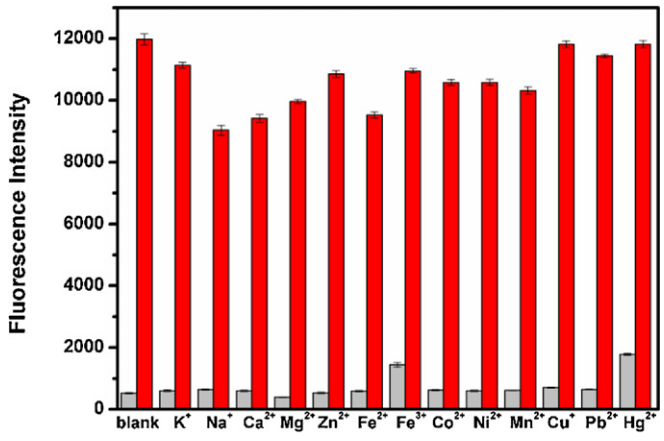

Fig. 2. Fluorescence intensity of RHAT (15 μM) in the presence of various cations at 576 nm. Gray bars represent the addition of an excess of the appropriate metal ion (1.0 mM for Na^+, K^+, 500 μM for Ca^{2+}, Mg^{2+} and Zn^{2+}, 25 μM for Fe^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Mn^{2+} and 5.0 μM for Cu^+, Pb^{2+}, Hg^{2+}) to a 5.0 μM solution of RHAT. Red bars represent the subsequent addition of 5.0 μM Cu^{2+} to the solution. Excitation was provided at 555 nm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the probe was stored for a long time. Fig. S5 displayed Cu^{2+} could be released from the complex upon addition of the chelating agent N,N,N′,N′-9-tetra (2-picolyl) ethylenediamine (TPEN).

3.5. The ability to accumulate into lysosomes

Considering spectroscopic data establishing that the probe can selectively respond to Cu^{2+} in aqueous solution, we evaluated RHAT in live-cell imaging assays in live human normal liver cells (HL-7702) and human liver carcinoma cell line (HepG2). First, we tested the probe for its ability to accumulate into lysosomes. Co-staining experiments with RHAT (Figs. 3a and S8a) and Lyso-Tracker DND-26, a commercially available lysosomal marker (Figs. 3b and S8b), showed that the observed fluorescence from RHAT is localized to lysosomes in these live cell lines (Figs. 3c and S8c).

3.6. Images of Cu^{2+} in different extent

We next investigated whether the probe is sensitive enough to detect labile lysosome Cu^{2+} and whether it can respond to increases and/or decreases of this Cu^{2+} pool [16]. HL-7702 cells incubated only with RHAT (15 μM) show very weak fluorescence (Fig. 4a). In contrast, when the cells were treated with 30 μM Cu^{2+} to raise global intracellular copper stores and subsequently imaged by confocal microscopy, as seen with HL-7702 cells, RHAT fluoresced brightly (Fig. 4b). When the cells were treated further by membrane-permeable chelator TPEN, a significant fluorescence decrease from the intracellular region was observed (Fig. 4c). Control experiments demonstrate the probe bound Cu^{2+} reversibly.

3.7. RHAT distinguish Cu^{2+} levels between normal liver and human hepatoma cells

Then we sought to assess whether the probe could show changes in the levels of labile Cu^{2+} in living cells. Ascorbate has been reported to facilitate the ceruloplasmin-dependent uptake and distribution of Cu^{2+} in K562 cells [17]. We reasoned that application of this reductant would shift the $Cu^{2+/+}$ redox equilibrium and increase the kinetically labile Cu^+ pool [7]. A marked fluorescence reduction is observed from Fig. 5b compared to Fig. 5a. The fluorescence intensity changes reveal that ascorbate-reduced samples possess a low level of labile Cu^{2+} compared to untreated samples. Similar results were obtained when adscititious Cu^{2+} was added (Fig. 5c and d).

To evaluate potential application of the fluorescent probe in the diagnosis and evaluation of copper-related diseases, sensor responsiveness was tested between live human normal liver cells

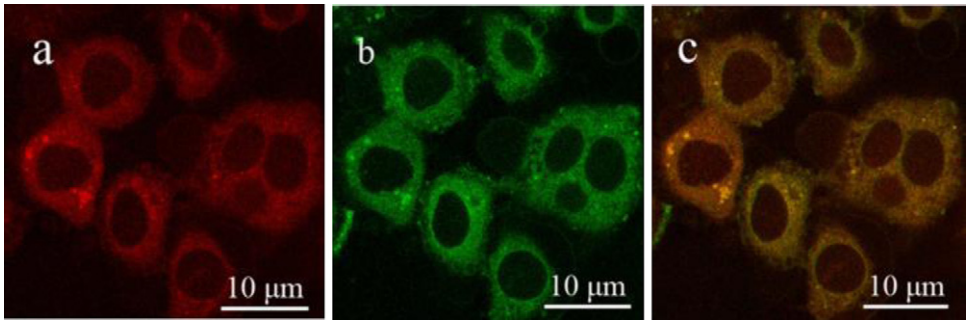

Fig. 3. (a) HL-7720 cells incubated with 30 μM Cu^{2+} for 30 min at 37 °C, then 15 μM RHAT for 15 min. (b) Addition of 50 nM Lyso-Tracker DND-26 for 10 min. (c) Overlay of (a) and (b).

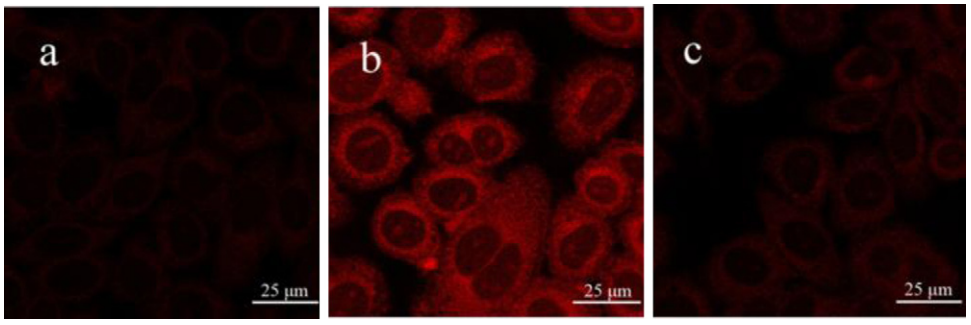

Fig. 4. Confocal fluorescence images of live HL-7702 cells. (a) Cells incubated with 15 μM RHAT for 15 min at 37 °C. (b) The cells were pretreated with 30 μM Cu^{2+} for 30 min at 37 °C, then were incubated with 15 μM RHAT for 15 min. (c) RHAT-supplemented cells pretreated with 30 μM Cu^{2+}, then treated with 50 μM TPEN.

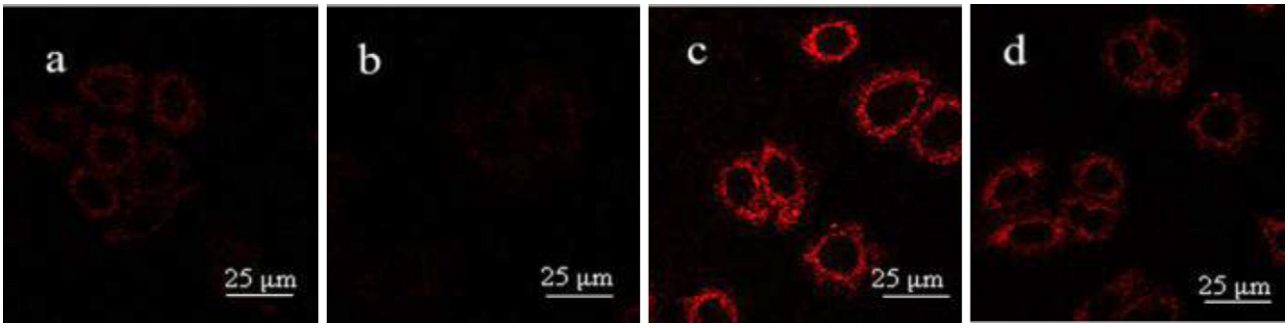

Fig. 5. (a) Confocal fluorescence images of live HL-7702 cells stained with 15 μM RHAT for 15 min at 37 °C. (b) HL-7702 cells treated with 50 μM ascorbate for 1 h and stained with 15 μM RHAT for 15 min at 37 °C. (c) HL-7702 cells treated with 30 μM Cu^{2+} for 30 min at 37 °C, then were incubated with 15 μM RHAT for 15 min. (d) HL-7702 cells treated with 30 μM Cu^{2+} for 30 min and 50 μM ascorbate for 1 h, then stained with 15 μM RHAT for 15 min at 37 °C.

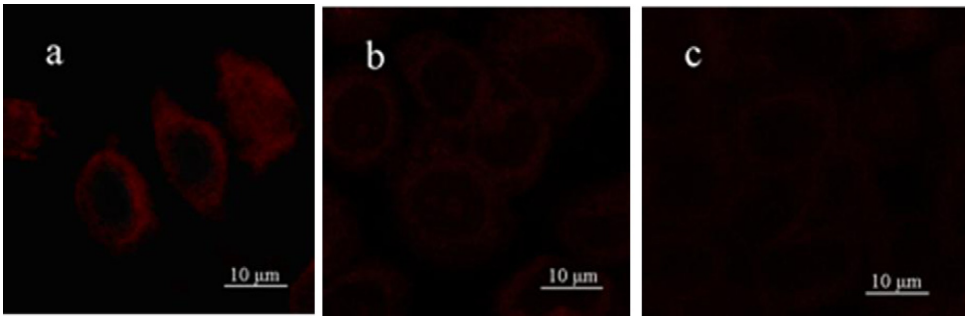

Fig. 6. (a) Confocal fluorescence images of live HepG2 cells grown in basal media and stained with 15 μM RHAT for 15 min at 37 °C. (b) HL-7702 cells with the same condition (a). (c) HL-7702 cells from condition (b) treated with 50 μM TPEN for 30 min.

(HL-7702) and human hepatoma cells (HepG2). As expected, we found that the fluorescence of Fig. 6a is apparently brighter than Fig. 6b. This means that the copper level within lysosomes of human hepatoma cells is higher to human normal liver cells, consistent with previously reported results. On the basis of the experiments, they will significantly contribute to ascertain the relationship between the concentrations of Cu^{2+} in lysosomes and the development of liver injury in living cells. On the other hand, compared with Fig. 6b and c, the results demonstrate that RHAT could detect intracellular Cu^{2+}. Additionally, RHAT displays low cytotoxicity under the experimental conditions via MTT assay (Fig. S10). Moreover, the results also demonstrate that RHAT had good membrane permeability to different cell types.

4. Conclusion

In closing, we have presented the synthesis, properties and biological applications of a new fluorescent probe to monitor Cu^{2+} concentration fluctuations in living cells. As described above, the probe can target specifically to lysosomes without the need for additional targeting groups. Additionally, the probe has good cell membrane permeability and low toxicity. The living cells imaging experiments show that the probe is capable of detecting changes in levels of intracellular Cu^{2+} upon exogenous copper addition, as well as sensing ascorbate-reduced decrease of endogenous Cu^{2+} pools concentration, distinguishing normal and cancer liver cells. The results also provide a new approach for potential biological and clinical implications.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jphotochem.2012.08.020.

References

[1] K. Glunde, C.A. Foss, T. Takagi, F. Wildes, Z.M. Bhujwalla, Synthesis of 6′-O-lissamine- rhodamine B-glucosamine as a novel probe for fluorescence imaging of lysosomes in breast tumors, Bioconjugate Chemistry 16 (2005) 843–851.
[2] M.S. Marks, P.A. Roche, E. van Donselaar, L. Woodruff, P.J. Peters, J.S. Bonifacino, A lysosomal targeting signal in the cytoplasmic tail of the b chain directs HLA-DM to MHC class II compartments, The Journal of Cell Biology 15 (1995) 351–369.
[3] S.C. Marks, Osteoclast biology: lessons from mammalian mutations, American Journal of Medical Genetics 34 (1989) 43–54.
[4] C.A. Renfrew, A.L. Hubbard, Degradation of epidermal growth factor receptor in rat liver. Membrane topology through the lysosomal pathway, The Journal of Biological Chemistry 266 (1991) 21265–21273.
[5] D. Klein, J. Lichtmannegger, U. Heinzmann, J. Müller-Höcker, S. Michaelsen, K.H. Summer, Association of copper to metallothionein in hepatic lysosomes of Long-Evans cinnamon (LEC) rats during the development of hepatitis, European Journal of Clinical Investigation 28 (1998) 302–310.
[6] M. Royzen, Z. Dai, J.W. Canary, Ratiometric displacement approach to Cu(II) sensing by fluorescence, Journal of the American Chemical Society 127 (2005) 1612–1613.
[7] S.J. Hyo, S.K. Pil, W.L. Jeong, K. Jae, S.H. Chang, W.K. Jong, Y. Shihai, Y.L. Jin, H.L. Jung, T. Joo, S.K. Jong, Coumarin-derived Cu^{2+}-selective fluorescence sensor: synthesis, mechanisms, and applications in living cells, Journal of the American Chemical Society 131 (2009) 2008–2012.

[8] L. Zeng, E.W. Miller, A.E. Pralle, Y. Isacoff, C.J. Chang, A selective turn-on fluorescent sensor for imaging copper in living cells, Journal of the American Chemical Society 128 (2006) 10–11.
[9] S.C. Dodani, S.C. Leary, P.A. Cobine, D.R. Winge, C.J. Chang, A targetable fluorescent sensor reveals that copper-deficient SCO1 and SCO2 patient cells prioritize mitochondrial copper homeostasis, Journal of the American Chemical Society 133 (2011) 8606–8616.
[10] P. Li, X. Duan, Z.Z. Chen, Y. Liu, T. Xie, L. Fang, X. Li, M. Yin, B. Tang, A near-infrared fluorescent probe for detecting copper(II) with high selectivity and sensitivity and its biological imaging applications, Chemical Communications 47 (2011) 7755–7757.
[11] S. Fakih, M. Podinovskaia, X. Kong, H.L. Collins, U.E. Schaible, R.C. Hider, Targeting the lysosome: fluorescent iron(III) chelators to selectively monitor endosomal/lysosomal labile iron pools, Journal of Medicinal Chemistry 51 (2008) 4539–4552.
[12] Y. Urano, D. Asanuma, Y. Hama, Y. Koyama, T. Barrett, M. Kamiya, T. Nagano, T. Watanabe, A. Hasegawa, P.L. Choyke, H. Kobayashi, Selective molecular imaging of viable cancer cells with pH-activatable fluorescence probes, Nature Medicine 15 (2009) 104–109.
[13] H.A. Benesi, J.H. Hildebrand, A spectrophotometric investigation of the interaction of iodine with aromatic hydrocarbons, Journal of the American Chemical Society 71 (1949) 2703–2707.
[14] M. Barra, C. Bohne, J.C. Scaiano, Effect of cyclodextrin complexation on the photochemistry of xanthone. Absolute measurement of the kinetics for triplet-state exit, Journal of the American Chemical Society 112 (1990) 8075–8079.
[15] E. Holtzmann, Lysosomes, Plenum Press, New York, London, 1989, 456 pp.
[16] D.W. Domaille, L. Zeng, C.J. Chang, Visualizing ascorbate-triggered release of labile copper within living cells using a ratiometric fluorescent sensor, Journal of the American Chemical Society 132 (2010) 1194–1195.
[17] E.D. Harris, S.S. Percival, A role for ascorbic acid in copper transport, The American Journal of Clinical Nutrition 54 (1991) 1193S–1197S.