NIH Public Access
Author Manuscript
Chem Commun (Camb). Author manuscript; available in PMC 2011 July 7.

Published in final edited form as:
Chem Commun (Camb). 2010 July 7; 46(25): 4472–4474. doi:10.1039/c000254b.

A novel profluorescent probe for detecting oxidative stress induced by metal and H_2O_2 in living cells

Yibin Wei, **Yi Zhang**, **Zhiwei Liu**, and **Maolin Guo**
Department of Chemistry and Biochemistry, University of Massachusetts, Dartmouth, MA 02747 (USA)

Maolin Guo: mguo@umassd.edu

Abstract

A profluorescent probe that has no fluorescent response to H_2O_2, iron or copper ions but can be readily activated in the presence of both H_2O_2 and Fe (or Cu) ion has been developed; the probe is capable of detecting oxidative stress promoted by Fe (or Cu) and H_2O_2 (i.e. the Fenton Reaction conditions) in living cells.

Oxidative stress plays a major role in the pathogenesis of a large number of human diseases, among which, the highly reactive and deleterious oxidizing species produced via the reaction of endogeneous H_2O_2 and redox metals (e.g., iron and copper) (the Fenton reactions (eq. 1)) have been implicated in the pathogenesis of Wilson's disease, Parkinson's disease (PD), Alzheimer's disease (AD), atherosclerosis, hemochromatosis, liver damage, cancer and diabetes, *etc*.1 Abnormal accummulation of redox metals (e.g., iron and copper) and overproduction of H_2O_2 in certain tissues in the body have been observed in patients with neurodegenerative diseases.1 Elevated levels of redox metal ions and H_2O_2 and the Fenton reactions contribute to the oxidative stress and neurodegeneration. We and others have been developing agents capable of performing H_2O_2-triggered "anti-Fenton reaction" via a prochelator activation and a subsequent metal caging strategy.2,3 The advantage of this novel strategy is that chelation can only be triggered by toxic levels of H_2O_2 thus will not interfere with the healthy metal homeostasis, holding promise in combating these diseases. However, a direct demonstration of the mechanism of this novel strategy in living systems is still lacking.

$$Fe^{II}(Cu^{I})+H_2O_2 \rightarrow Fe^{III}(Cu^{II})+HO^{-}+HO\bullet \quad (1)$$

To provide direct evidence on the mechanism of this novel anti-Fenton strategy in living systems, we have developed our third generation prochelator, **RS-BE**, which is profluorescent and does not react with Fe (or Cu) ions. However, it can be converted to an active chelator by H_2O_2, still fluorescence silent, but the fluorescence is activated upon metal chelation on the active chelator. Thus, the "anti-Fenton process" is readily monitored by an ideal "turn-on" fluorescent process in living cells which is described in this communication.

The profluorescent prochelator **RS-BE** was designed by "masking" the key chelating hydryoxyl group in the active chelator **Rh-SBH** with a bulky boronic acid pinacol ester

This journal is © The Royal Society of Chemistry [year]
Correspondence to: Maolin Guo, mguo@umassd.edu.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

group which may be unmasked by H_2O_2 (Scheme 1). Subsequent metal coordination with the active chelator **Rh-SBH** may induce the conversion of the profluorescent **Rh-SBH** molecule from the ring-closed spirolactam form (non-fluorescent) to the ring-opened amide form (fluorescent) in the metal complexes (Scheme 1).4 This metal-coordination induced profluorescence activation process may be called a “coordination-induced fluorescence activation (CIFA)”.

Synthesis of the prochelator **RS-BE** was accomplished in 3 0 % yield by refluxing rhodamine hydrazine and 2-fomylphenylboronic acid, pinacol ester in ethanol (ESI†). The active chelator, **Rh-SBH**, was also synthesized and characterized following a published procedure. 4

The UV-vis spectroscopic properties of **RS-BE** and its interactions with Cu^{2+}, Fe^{2+} and H_2O_2 were evaluated first. Due to limited water solubility of **RS-BE**, a mixed solvent acetonitrile(ACN)/potassium phosphate buffer (KPB) (10 mM, pH 7.5, v/v 1:1) was used. Solution of **RS-BE** (50 μM) is colorless, exhibiting absorption in UV region (240–300 nm) only (Fig.S1). As shown in Fig.1 and Fig. S1, addition of Cu^{2+} or Fe^{2+} ions changes little the aborption characteristics of **RS-BE**, suggesting the prochelator does not bind strongly to Cu^{2+} or Fe^{2+} ions under the conditions. However, upon addition of excess H_2O_2 to the systems (60 min incubation with 500 μM H_2O_2), the solutions turned pink and a new peak at 550 nm was observed in both the Cu^{2+}- and Fe^{2+}-**RS-BE** systems (Fig.1 and Fig. S1; Fe^{2+} may be oxidized to Fe^{3+} by excess H_2O_2 here), implying metal-chelation occurred via a H_2O_2-triggered prochelator activation (from **RS-BE** to **Rh-SBH)** and subsequent metal-chelation mechanism (Scheme 1), as demonstrated for our previously designed prochelators. 2 Further investigations on the interactions of Fe^{2+} or Fe^{3+} with the active chelator **Rh-SBH** suggest that Rh-SBH response to Fe^{3+}, not Fe^{2+}(Fig. S2). A strong response of Rh-SBH to Cu^{2+} but a weaker response to Fe^{2+} were also reported under aerobic conditions in a different buffer (ACN/Tris, 10 mM, pH 7.0).4 The appearance of the new peak at 550 nm has been assigned to the metal-binding induced conversion from ring-closed spirolactam form of **Rh-SBH** (colourless) to the ring-opened amide form (pink) in the metal complexes. 4 Rh-SBH displays a selective absorption response to Cu^{2+} and Fe^{3+} over other metal ions (Fig. S3), and the spirolactam form is stable over pH 5.0 to 8.2, in agreement with that reported.4

The clean conversion of **RS-BE** to **RH-SBH** by H_2O_2 was further confirmed via NMR spectroscopy. As shown in Fig. 2, after incubating **RS-BE** with H_2O_2, ^{1}H NMR peaks for **RS-BE** (δ 9.42(s), =CH-) gradually decreased in intensity, while the peaks corresponding to **Rh-SBH** (δ 9.09(s), =CH-; 10.45(s) – OH−) appeared simultaneously and increased in intensity with time. The peaks (δ7.81(d), 7.51–7.57(m) and 7.30(t), salicylaldehyde) also underwent similar conversions. Meanwhile, the peaks for boric acid and pinacol, the H_2O_2-deprotected products of the boronic acid pinacol ester moiety, appeared at δ6.55(s, $B(OH)_3$), δ7.99(s, -OH) and δ1.15 (s, 4×(CH_3), Figs. S5, S6). No ^{1}H NMR change was observed for the xanthene moiety due to its distance from the reaction site. In ~2 h, **RS-BE** had been cleanly converted to **Rh-SBH** with no intermediate formed, as indicated by the ^{1}H-NMR spectra. ^{13}C-NMR data also confirmed that **RS-BE** was converted by H_2O_2 to **Rh-SBH** which is still in the the ring-closed spirolactam form (Fig. S7).

We further examined the fluorescence responses of **RS-BE** to Cu^{2+}, Fe^{2+} and H_2O_2. As shown in Fig. 3, **RS-BE** in ACN/KPB buffer (1:1, pH 7.5) exhibits weak fluoresence at 575 nm (curve (a)). The fluorescence is not affected by the presence of H_2O_2 (Fig. S8). Addition

†Electronic supplementary information(ESI) avaivable: details on synthesis, absorption, NMR and fluorescence spectra, cell culture and imaging.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

of Cu^{2+} or Fe^{2+} (curves (b) in Fig. 3) to **RS-BE** in the absence of H_2O_2 did not change its fluorescence profiles either. However, upon the addition of H_2O_2 to the system containing **RS-BE**/Cu^{2+}(or Fe^{2+}), as we expected, the fluorescent intensity at 575 nm increased with time (curves (c)–(g) in Fig.3 (I) and (c)–(f) in Fig. 3 (II)). At 2 h, a ~7.5-fold increase in fluorescent intensity was observed in the **RS-BE**/Cu^{2+}/H_2O_2 system while a ~5-fold increase in the **RS-BE**/Fe^{2+}/H_2O_2 system (Fe^{2+} may be oxidized to Fe^{3+} by excess H_2O_2 here). In addition, the emission spectrum of **RS-BE**/Cu^{2+}(or Fe^{2+}) underwent a red shift from 575 nm to 580 nm after the addition of H_2O_2. This fluorescence response matches those observed for the reactions of **Rh-SBH** with metal ions.4 Therefore, we can conclude that in the presence of Cu^{2+}(or Fe^{2+}), the fluorescence enhancement of **RS-BE** upon additon of H_2O_2 must be occurred via a H_2O_2-triggered prochelator activation (from **RS-BE** to **Rh-SBH)** and subsequent metal-chelation mechanism (Scheme 1), corroborating the conclusions from the UV-vis studies (Fig. 1). These interesting fluorescent responses offer us the opportunity to study the “anti-Fenton” mechanism in living cells by using **RS-BE** as a novel “turn-on” fluorescent probe.

RS-BE was then tested in live cells (SH-SY5Y neuroblastoma cells, a human neuronal cell line) its fluorescent responses to Cu^{2+}, Fe^{3+} and H_2O_2 via a laser scanning confocal microscope (Zeiss LSM 710). Iron- and copper-8-hydroxylquinoline complexes (Fe(8-HQ) and Cu(8-HQ)) were used to enhance membrance permeability of the metal ions.5,6 Human SH-SY5Y cells loaded with 10 μM **RS-BE** (incubated for 30 min) did not show intracellular fluoresence (Fig. 4(b)). Then, the cells were further implemented with 10 μM Fe(8-HQ). No fluorescence was observed after 30 min incubation (Fig. 4(c)), suggesting that the normal cellular components or the added Fe(8-HQ) does not trigger fluorescent signal of the profluorescent probe **RS-BE**, as we desired. At last, we treated the Fe(8-HQ) loaded cells with 100 μM H_2O_2, followed by immediate recording fluorescence images of the cells every 5 mins for 30 min. Excitingly, as shown in Fig. S9 and Fig. 4(d) and (e), intracellular fluorescent signal which matches the profile of **Rh-SBH**-metal comlexes emerged after 5 min treatment with H_2O_2 and increased in intensity with time. A ~10-fold increase in intensity was observed after 25 min (Fig.4(f)). These data suggest that a H_2O_2-triggered prochelator activation (from **RS-BE** to **Rh-SBH)** followed by Fe-chelation occurred in the cells.

Similar experiements were performed to test the response of **RS-BE** with Cu^{2+} and H_2O_2 in human SH-SY5Y cells. As expected, supplement of 10 μM **RS-BE** and subsequent 10 μM Cu(8-HQ) or $CuCl_2$ did not result in an intracellular fluorescent response (Fig.5(b) and (c), Fig. S10). Further addition of 100 μM H_2O_2 to the cells loaded with **RS-BE** and Cu(8-HQ) displayed a specific intracellular fluorescence enhancement (Fig. 5(d) and (e)) but with weaker intensity compared to those with Fe^{3+}. In 60 min, only ~3.8-fold increase in intensity was observed. Little intracellular fluorescence enhancement was observed 30 min after the addition of 100 μM H_2O_2 to the cells preloaded with **RS-BE** and $CuCl_2$ (Fig. S10). However, cells treated with higher levels of H_2O_2 (500 μM) and $CuCl_2$ (50 μM) did exhibit the specific intracellular fluorescence enhancement, though still weak (Fig. S11). The weaker fluorescent response in the cells loaded with Cu^{2+} may be due to poor availability of free copper ions in cells.7

Taken together, we have developed a novel prochelator-type profluorescent sensor **RS-BE** that does not have fluorescent response to H_2O_2, iron or copper ions but the fluorescence can be readily “turned-on” in the presence of both H_2O_2 and Fe (or Cu) ions. The sensor works *via* a H_2O_2- triggered prochelator activation (from **RS-BE** to **Rh-SBH)** followed by metal-coordination-induced fluorescence activation (CIFA) mechanism, thus providing an ideal probe to monitor the “anti-Fenton” processes by a “turn-on” fluorescence process. This sensor has been demonstrated the ability to monitor the *in situ* presence of H_2O_2 and Fe (or

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Cu) ions (i.e. the “Fenton Reaction” conditions) in live human SH-SY5Y cells, therefore, is capable of detecting oxidative stress promoted by H_2O_2 and Fe (or Cu) in cellular system. However, upon being “turned on”, this sensor cannot signal the subsequent drop in H_2O_2 levels as the reaction of RS-BE with H_2O_2 to generate **Rh-SBH** is irreversible.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank the University of Massachusetts, Dartmouth and the National Institutes of Health (Grant No. 1 R21 AT002743-02 from the National Center for Complementary and Alternative Medicine (NCCAM)) for funding.

Notes and References

1. (a) Crichton, R. Inorganic Biochemistry of Iron Metabolism. West Sussex: John Wiley & Sons, Ltd; 2001. (b) Barnham KJ, Masters CL, Bush AI. Nat. Rev. Drug Discov. 2004; 3:205–214. [PubMed: 15031734] (c) Zecca L, Stroppolo A, Gatti A, et al. PNAS. 2004; 101:9843–9848. [PubMed: 15210960] (d) Perez C, Tong Y, Guo M. Curr. Bioactive Comp. 2008; 4:150–158. (e) Chaston TB, Richardson DR. Am. J. Hematol. 2003; 73:200–210. [PubMed: 12827659] (f) Krapfenbauer K, Engidawork E, Cairns N, Fountoulakis M, Lubec G. Brain. Res. 2003; 967:152–160. [PubMed: 12650976] (g) Tabner BJ, Turnbull S, El-Agnaf OMA, Allsop D. Free Rad. Biol. Med. 2002; 32:1076–1083. [PubMed: 12031892] (h) Cooper GJS, Young AA, Gamble GD, et al. Diabetologia. 2009; 52:715–722. [PubMed: 19172243]
2. (a) Wei Y, Guo M. Angew. Chem. Int. Ed. 2007; 46:4722–4725. (b) Wei Y, Guo M. Chem. Commun. 2009:1413–1415.
3. Charkoudian LK, Pham DM, Franz KJ. J. Am. Chem. Soc. 2006; 128:12424–12425. [PubMed: 16984186]
4. Xiang Y, Tong A, Jin P, Yong J. Org. Lett. 2006; 8:2863–2866. [PubMed: 16774276]
5. Berchner-Pfannschmidt U, Petrat F, Doege K, et al. J. Biol. Chem. 2004; 279:44976–44986. [PubMed: 15322093]
6. Daniel KG, Gupta P, Harbach RH, Guida WC, Dou QP. Biochem. Pharmacol. 2004; 67:1139–1151. [PubMed: 15006550]
7. Kim B-E, Nevitt T, Thiele DJ. Nat. Chem. Biol. 2008; 4:176–185. [PubMed: 18277979]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

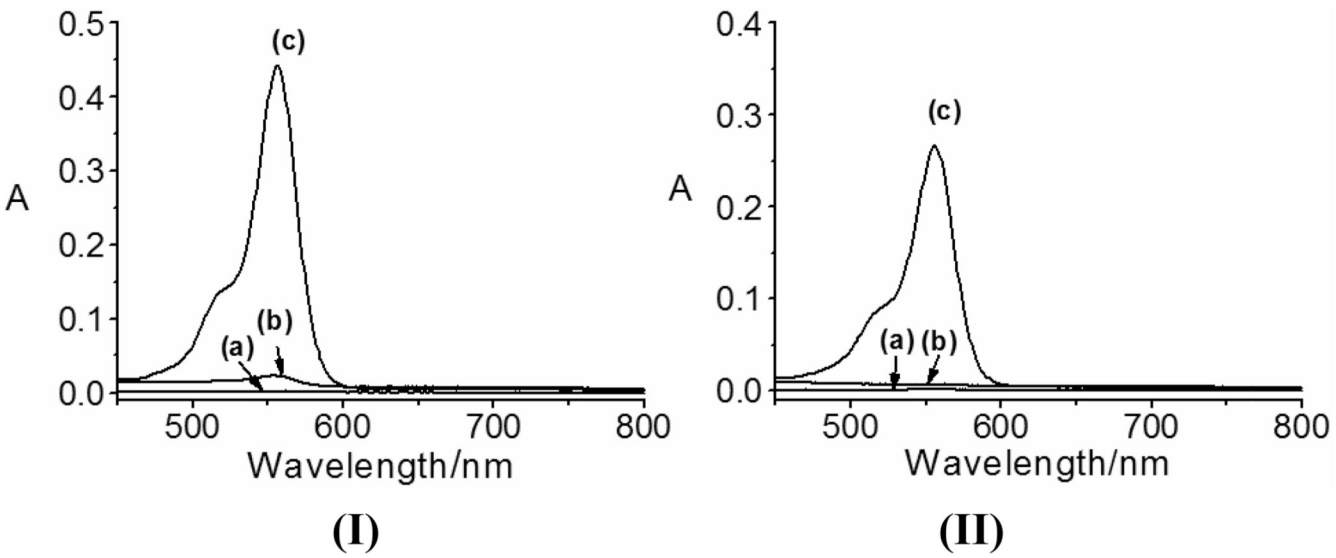


Fig. 1.

Absorption spectra of **RS-BE** and its interactions with Cu^{2+} **(I)** or Fe^{2+} **(II)** before and after addition of 500 μM H_2O_2 in ACN/KPB buffer (10 mM, pH 7.5, v/v 1:1): (a) **RS-BE** (50 μM) only, (b) **RS-BE** with metal ions (50 μM) and (c) with metal ions and H_2O_2 (500 μM).

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

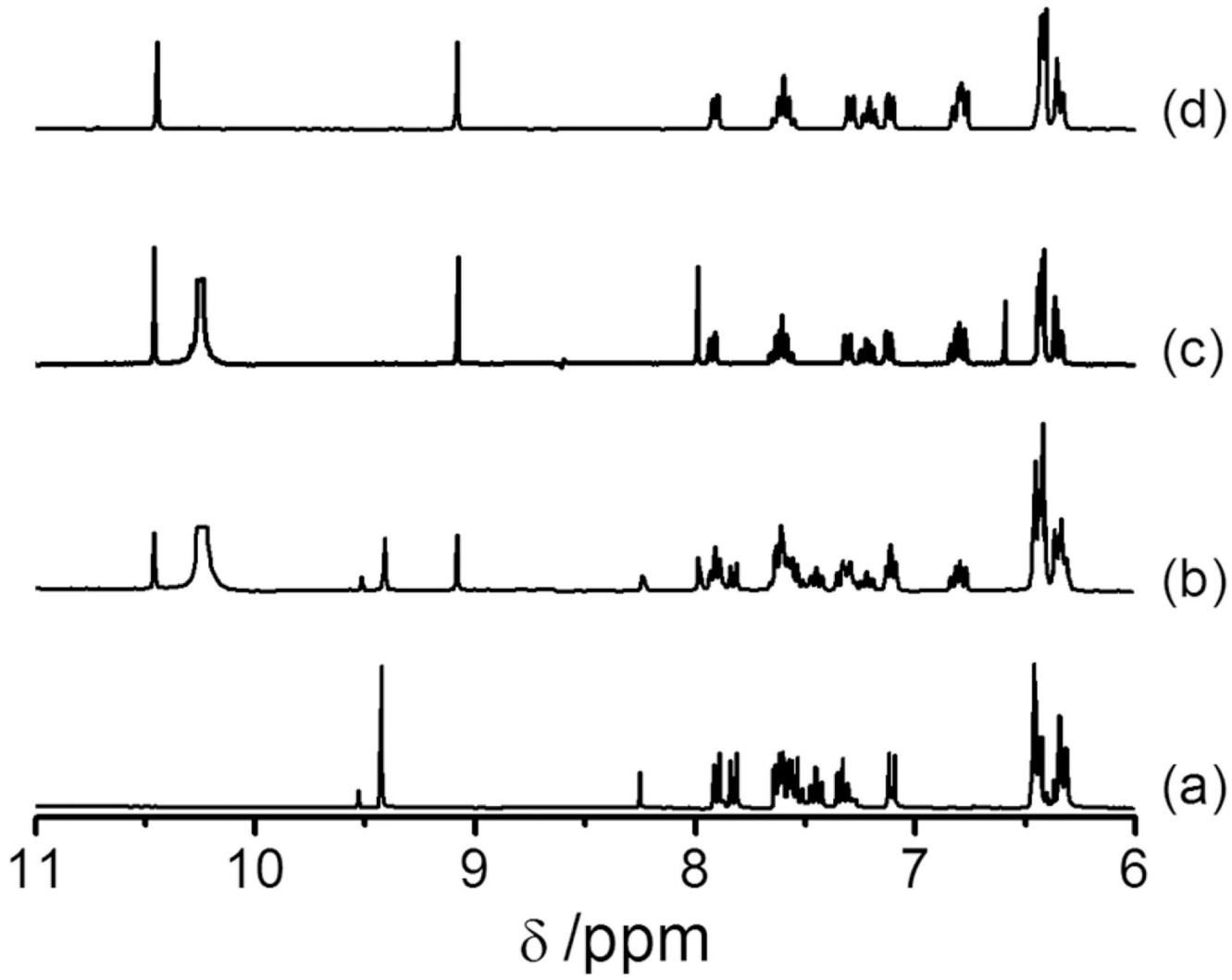


Fig. 2.

^{1}H NMR spectra (from δ11.0- 6.0, $(CD_3)_2SO$)) of (a) **RS-BE** (5 mM); (b) and (c), the reaction of **RS-BE** (5 mM) with H_2O_2 (50 mM) at 293 K after 1 h and 2 h, respectively; and (d) **Rh-SBH** (5 mM).

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

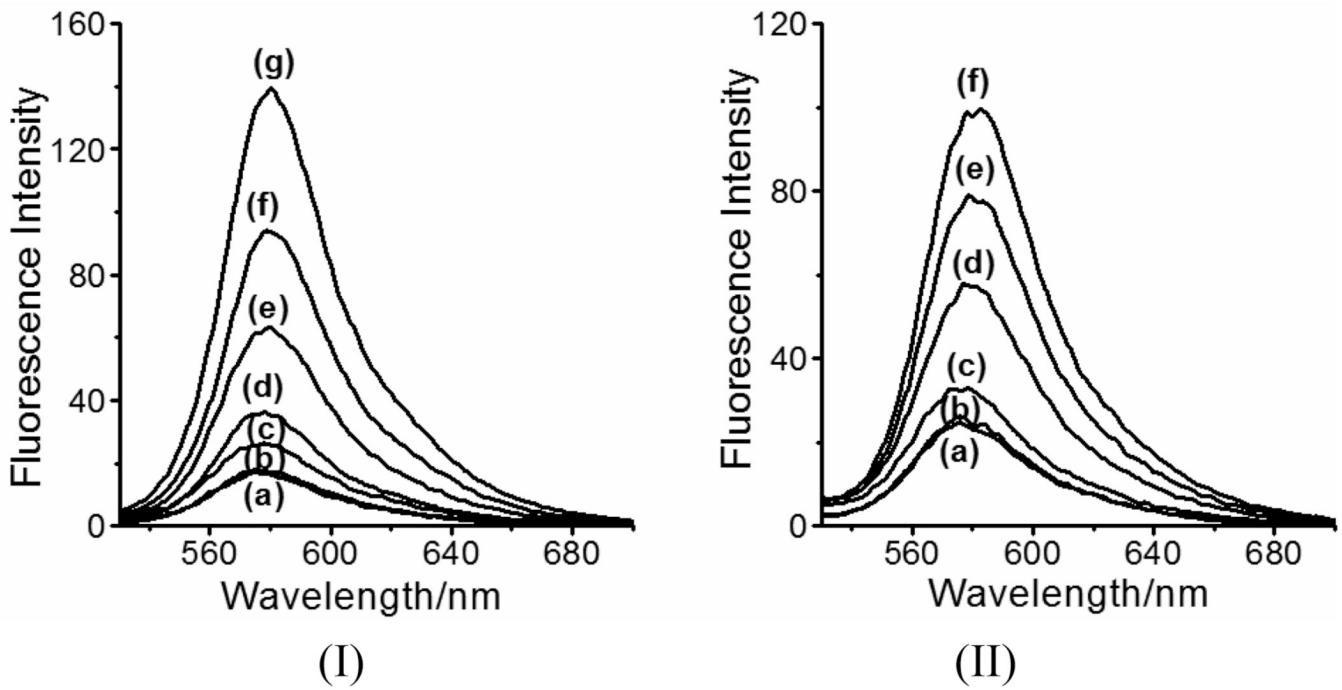


Fig. 3.

H_2O_2-triggered fluorescence responses (E_x, 510 nm; E_m, 580) of **RS-BE** (50 μM) to Cu^{2+} (I) or Fe^{2+} (II) (50 μM) in ACN/KPB buffer (10 mM, pH 7.5, v/v 1:1). (Ia) and (IIa) **RS-BE** only; (Ib) and (IIb) **RS-BE** with Cu^{2+} and Fe^{2+}, respectively; from (Ic) to (Ig), **RS-BE**/Cu^{2+} incubated with 500 μM H_2O_2 for 10, 30, 60, 90 and 120 min, respectively; from (IIa) to (IIf), **RS-BE**/Fe^{2+} incubated with 500 μM H_2O_2 for 30, 60, 90 and 120 min, respectively.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

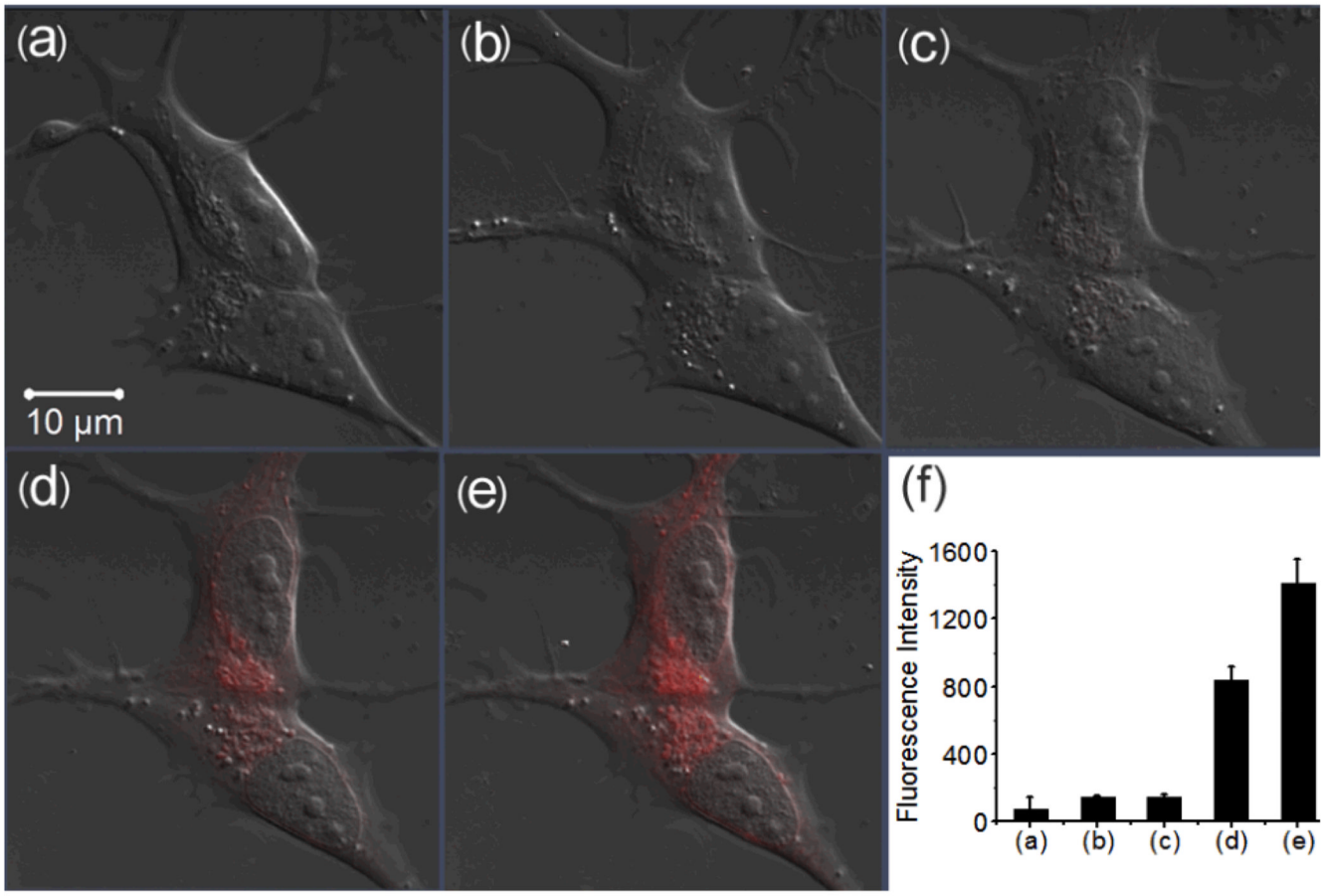


Fig. 4.

Confocal fluorescence images of live human SH-SY5Y cells with the treatment of **RS-BE**/ Fe/ H_2O_2 (scale bar 10 μm). (a) DIC; (b) the cells incubated with 10 μM **RS-BE** for 30 min; (c) the cells were then incubated with 10 μM Fe(8-HQ) for 30 min; (d) and (e) the cells were further treated with 100 μM H_2O_2 for 10 and 25 min, respectively, (f) Integrated emission (547–703 nm) intensity of (a), (b), (c), (d) and (e) images.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

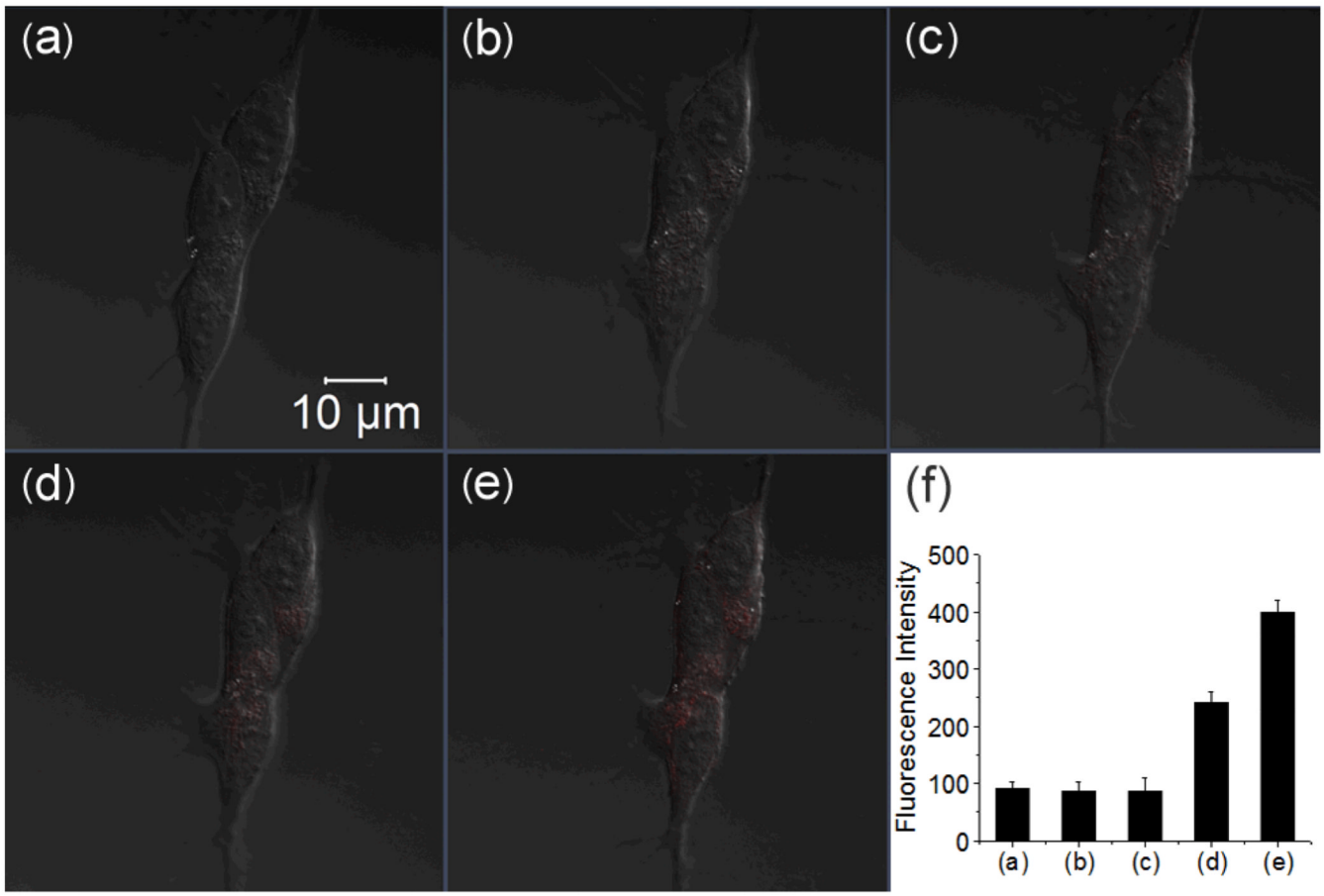


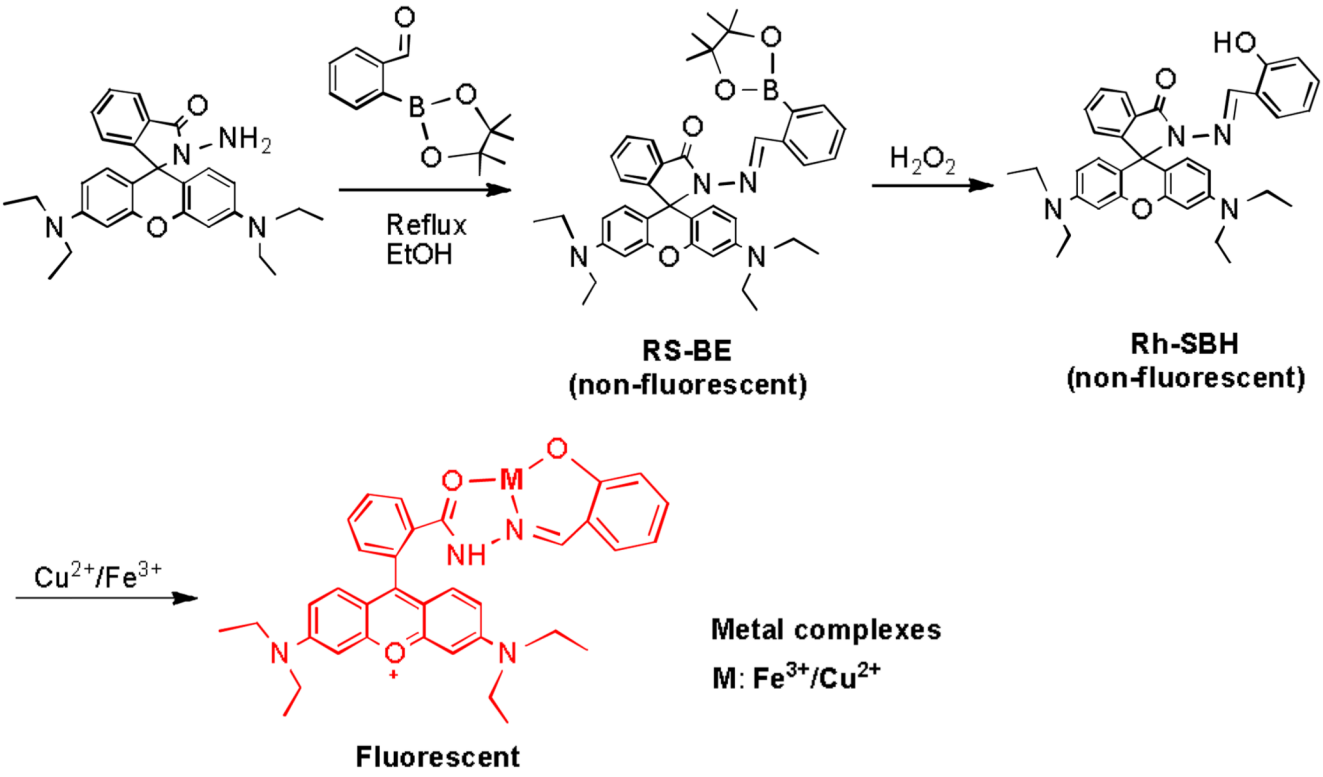
Fig. 5.

Confocal fluorescence images of live human SH-SY5Y cells with the treatment of **RS-BE**/ Cu/ H_2O_2 (scale bar 10 μm). (a) DIC; (b) cells incubated with 10 μM **RS-BE** for 30 min; (c) the cells were then incubated with 10 μM Cu(8-HQ) for 30 min; (d) and (e) the cells were further treated with 100 μM H_2O_2 for 30 and 60 min, respectively; (f) Integrated emission (547–703 nm) intensity of (a), (b), (c), (d) and (e) images.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript



Scheme 1.
Synthesis and activation of **RS-BE**