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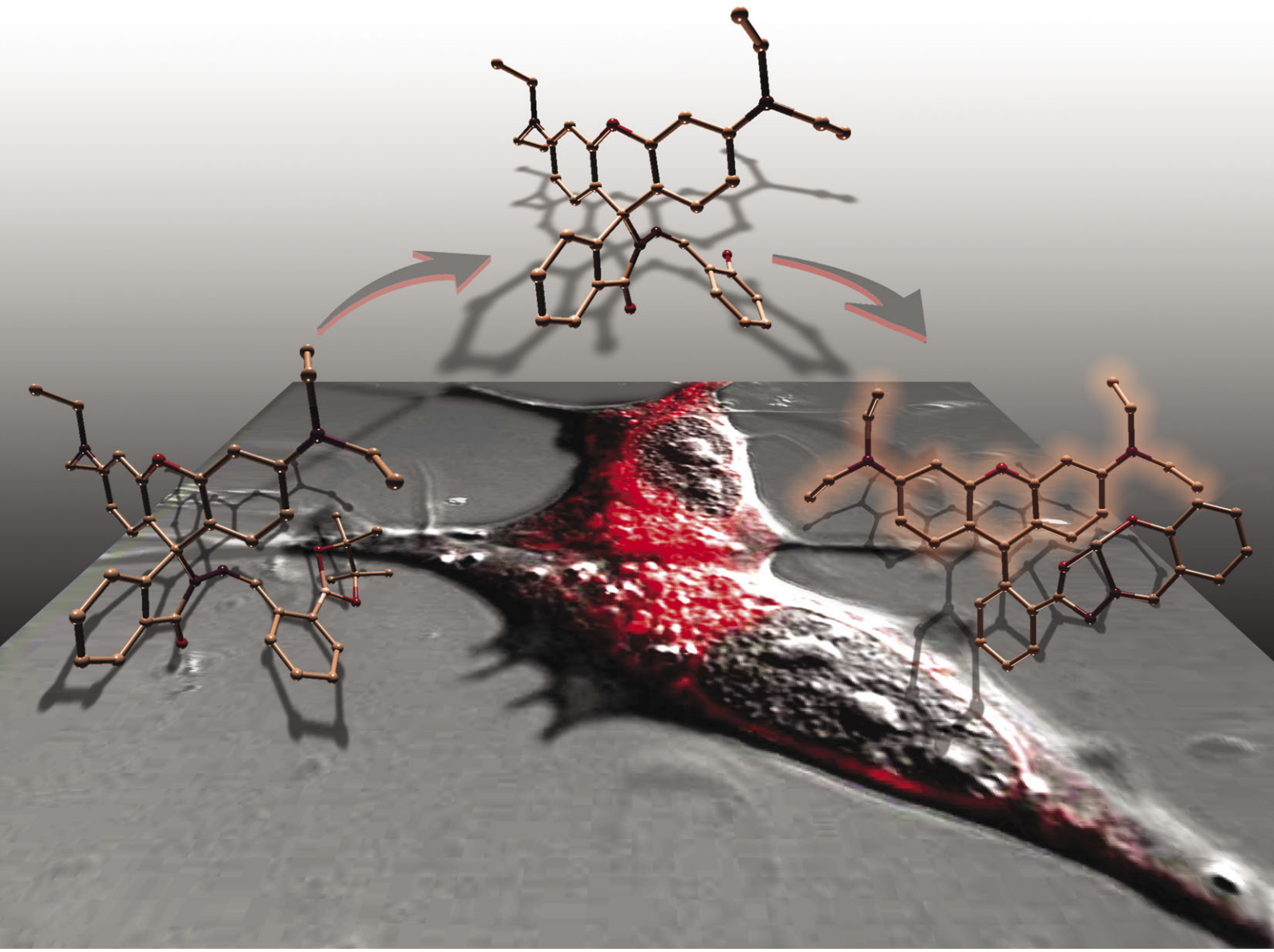

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A novel profluorescent probe for detecting oxidative stress induced by metal and H_2O_2 in living cells†

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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. 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 reaction (eqn (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.

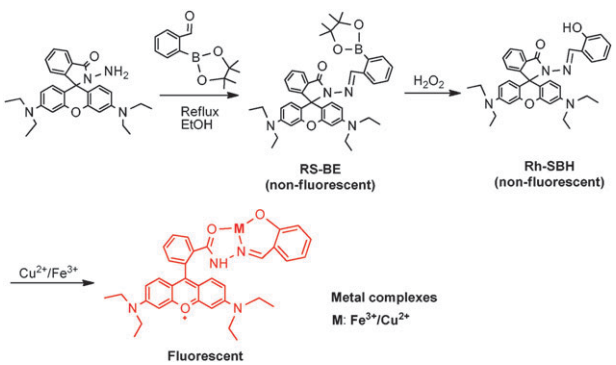
$$Fe^{II}\ (Cu^{I}) + H_2O_2 \rightarrow Fe^{III}(Cu^{II}) + HO^- + HO^\bullet \quad (1)$$

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 hydroxyl group in the active chelator **Rh-SBH** with a bulky boronic acid pinacol ester 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 30% yield by refluxing rhodamine hydrazine and 2-formylphenylboronic 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. The solution of **RS-BE** (50 μM) is colorless, exhibiting absorption in the 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 absorption 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 a 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** responds 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



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

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† Electronic supplementary information (ESI) available: Details of synthesis, absorption, NMR and fluorescence spectra, cell culture and imaging. See DOI: 10.1039/c000254b

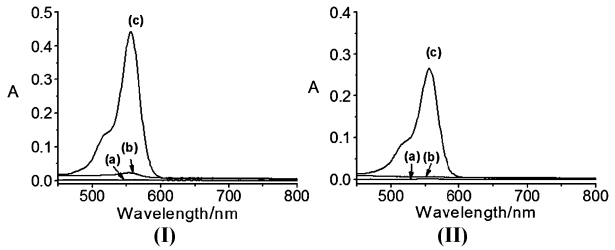

Fig. 1 Absorption spectra of **RS-BE** and its interactions with Cu^{2+} (I) or Fe^{2+} (II) before and after addition of 500 μM H_2O_2 in ACN/KPB buffer (10 mM, pH 7.5, v/v 1 : 1): (a) **RS-BE** (50 μM) only, (b) **RS-BE** with metal ions (50 μM) and (c) with metal ions and H_2O_2 (500 μM).

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 the 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 (δ 7.81(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 δ 6.55(s, $B(OH)_3$), δ 7.99(s, –OH) and δ 1.15 (s, 4 × (CH_3), Fig. S5 and 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 ring-closed spirolactam form (Fig. S7†).

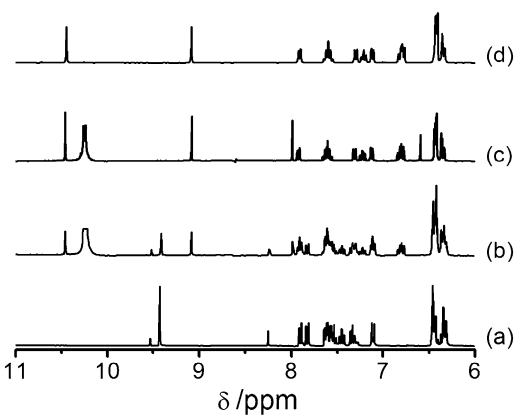

Fig. 2 ^{1}H NMR spectra (from δ 11.0–6.0, $(CD_3)_2SO$) of (a) **RS-BE** (5 mM); (b) and (c), the reaction of **RS-BE** (5 mM) with H_2O_2 (50 mM) at 293 K after 1 h and 2 h, respectively; and (d) **Rh-SBH** (5 mM).

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 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 was observed 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 addition of H_2O_2 must occur *via* a H_2O_2-triggered prochelator activation (from **RS-BE** to **Rh-SBH**) and a 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) for 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 membrane 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 fluorescence (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) do not trigger a fluorescent signal of the profluorescent probe **RS-BE**, as we desired. Lastly, we treated the Fe(8-HQ) loaded cells with 100 μM H_2O_2, followed by

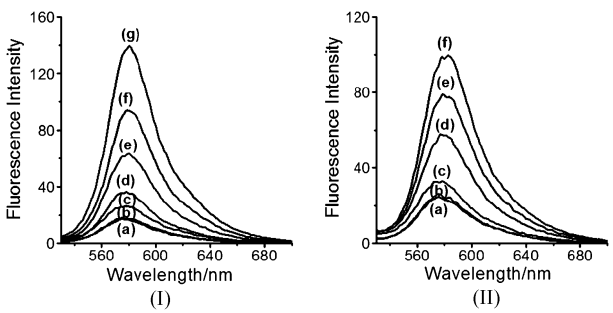

Fig. 3 H_2O_2-triggered fluorescence responses (E_x, 510 nm; E_m, 580) of **RS-BE** (50 μM) to Cu^{2+} (I) or Fe^{2+} (II) (50 μM) in ACN/KPB buffer (10 mM, pH 7.5, v/v 1 : 1). (Ia) and (IIa) **RS-BE** only; (Ib) and (IIb) **RS-BE** with Cu^{2+} and Fe^{2+}, respectively; from (Ic) to (Ig), **RS-BE**/Cu^{2+} 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.

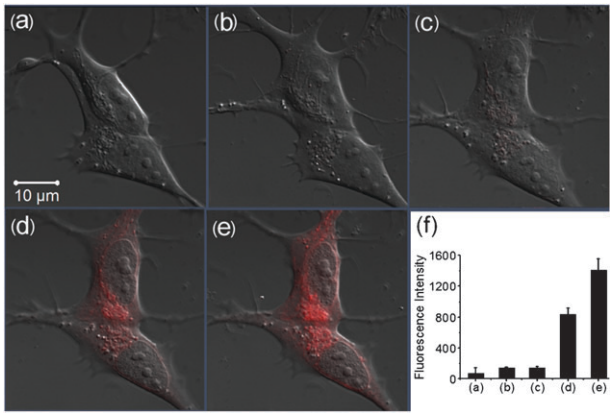

Fig. 4 Confocal fluorescence images of live human SH-SY5Y cells with treatment with **RS-BE**/Fe/H_2O_2 (scale bar 10 μm). (a) DIC; (b) the cells incubated with 10 μM **RS-BE** for 30 min; (c) the cells were then incubated with 10 μM Fe(8-HQ) for 30 min; (d) and (e) the cells were further treated with 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.

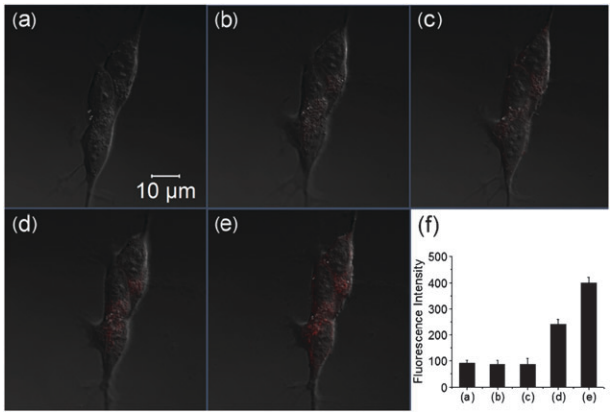

Fig. 5 Confocal fluorescence images of live human SH-SY5Y cells with treatment with **RS-BE**/Cu/H_2O_2 (scale bar 10 μm). (a) DIC; (b) cells incubated with 10 μM **RS-BE** for 30 min; (c) the cells were then incubated with 10 μM Cu(8-HQ) for 30 min; (d) and (e) the cells were further treated with 100 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.

immediate recording of the fluorescence images of the cells every 5 min for 30 min. Excitingly, as shown in Fig. S9† and Fig. 4(d) and (e), an intracellular fluorescent signal which matches the profile of **Rh-SBH**–metal complexes 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 experiments were performed to test the response of **RS-BE** with Cu^{2+} and H_2O_2 in human SH-SY5Y cells. As expected, a 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) resulted in a specific intracellular fluorescence enhancement (Fig. 5(d) and (e)) but with weaker intensity compared to those with Fe^{3+}. In 60 min, only a ~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]

In conclusion, we have developed a novel prochelator-type profluorescent sensor **RS-BE** that does not have a 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 a 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 demonstrated the ability to monitor the *in situ* presence of H_2O_2 and Fe (or Cu) ions (*i.e.* the "Fenton reaction" conditions) in live human SH-SY5Y cells, therefore it is capable of detecting oxidative stress promoted by H_2O_2 and Fe (or Cu) in a 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.

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