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Reversible and selective luminescent determination of ClO^-/H_2S redox cycle in vitro and in vivo based on a ruthenium trisbipyridyl probe

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A rapid and highly selective luminescent probe has been developed to determine the in vitro and in vivo ClO^-/H_2S redox cycle using a ruthenium *tris*-bipyridyl complex covalently linked with phenothiazine. The luminescence intensity was considerably enhanced upon the addition of ClO^- due to the oxidation of the probe to its sulfoxide derivative, which quickly returned to the original level by the reaction with H_2S due to the reconstitution of the probe. The redox cycle can be repeated at least 12 times. Under optimal conditions, the luminescence intensities are linear over the concentration range 1×10^{-9}–1×10^{-4} mol/L for ClO^- and 1×10^{-9}–1×10^{-4} mol/L for H_2S, and the detection limits are 1.8×10^{-11} mol/L for ClO^- and 1.2×10^{-11} mol/L for H_2S, which are much lower than those obtained with other detection methods. The proposed method is simple in design and fast in operation, and is suitable for the reversible determination of ClO^- and H_2S in vitro and in vivo with high selectivity.

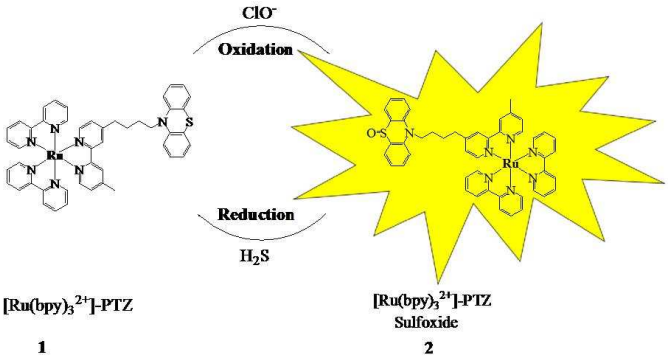
Introduction

Hypochlorite (ClO^-) is widely used as disinfector and decolorizer in daily applications. Moreover, it is an important reactive oxygen species (ROS), which has been known to be essential for several biological functions.[1-4] Endogenous ClO^- is produced by peroxidation between peroxide and chloride ions under catalization of the enzyme myeloperoxidase.[5,6] In cells, ClO^- plays a critical role in antibacterial processes, killing a wide range of pathogens.[3,7] However, excessive generation of ClO^- can cause oxidative damage of DNA and RNA, and even lead to various pathological diseases including cardiovascular disease,[8,9] neurodegeneration,[10] arthritis, and cancers.[11] Importantly, it gained much attention from medical scientists, biologists, biochemists, and environmental chemists for the development of a rapid and highly selective method to monitor ClO^-.

There are three gaseous signaling molecules in organs and cells, nitric oxide (NO), carbon monoxide (CO), and hydrogen sulfide (H_2S). Among them, H_2S plays a prominent role in physiology and pathophysiology.[12] H_2S is enzymatically generated in the metabolism of many organs (e.g., heart, liver, kidneys, brain, nervous system) and tissues (e.g., connective tissues, adipose tissues).[13] There is evidence that many chronic diseases are associated with abnormal levels of H_2S, such as gastric mucosal injury,[14] diabetes, cardiovascular disease, liver cirrhosis,[15] and Alzheimer's disease.[16] Therefore, new methods are also needed for the efficient detection of sulfide in biological systems.

Much effort has been focused on monitoring ClO^- and H_2S in biological systems. Among them, luminescence measurements have many advantages over other detection technologies, owing to higher sensitivity, better selectivity, and in situ observation. In recent years, several luminescent probes have been used to monitor ClO^- [17-23] and H_2S.[24-31] However, it should be noted that ClO^- and H_2S coexist in biological systems and the redox cycle between ClO^- and H_2S plays a prominent role in physiology and pathophysiology.[2,12] Therefore, developing a reversible and highly selective luminescent probe to monitor the ClO^-/H_2S redox cycle will be beneficial for fully understanding and treating numerous pathological diseases. For this purpose, it is necessary to find a redox reversible luminescent probe. Han et al. developed a smart probe (Se-BODIPY) for monitoring and visualizing the redox process between the oxidative stress of HClO and H_2S repair in living cells, depending on the redox chemistry of the versatile selenium.[32] The detection limits for HClO and H_2S were 0.98 and 1.08 μM under the test conditions.

Both S and Se belong to the elemental family of oxygen, and phenothiazine (PTZ) is a typical S-containing compound with a reversible one-electron oxidation potential.[33] Peng et al.[34] reported on a PTZ-Cy2 fluorescent probe to detect ClO^- in aqueous solution and living cells, suggesting that PTZ can be an efficient functional group. Unfortunately, cyanine dyes are not stable during redox processes. Ruthenium *tris*-bipyridyl complexes are good candidates owing to their reversible redox ability. In recent years, $Ru(bpy)_3^{2+}$ and its derivatives have been of particular interest for luminescence applications because of their low-lying metal-to-ligand charge transfer excited state, long-lived excited-state lifetimes, pronounced stability, and tunable luminescent color (emission around 610 nm). In our previous work,[33] $[Ru(bpy)_3^{2+}]$-PTZ (Scheme 1, **1**) was designed as an intramolecular electron transfer electrochemiluminescence label that exhibited good redox reversibility, suggesting a potential approach to reversibly monitor the ClO^-/H_2S redox cycle. To demonstrate this, **1** was used as a luminescent probe. As shown in Scheme 1, the luminescence intensity of **1** obviously increased in the presence of ClO^- due to the oxidation of the PTZ group to form **2**, and the increased luminescence intensity quickly returned to the original level in the presence of H_2S owing to the recovery of **1**. The measurement exhibits lower detection limits and can be repeated at least for 12 redox cycles.



Scheme 1 Structure of **1** and its schematic luminescence response to the redox cycle of ClO^- and H_2S.

Experimental section

Materials and Reagent

Hexafluorophosphate (PF_6^-) salt of **1** was a sample left from our previous work.[33] Compound **1** (1 mM) in 0.1 M phosphate-buffer saline (PBS; pH 7.4) was kept at 4 ºC before the analyses. ClO^- solution was standardized at pH 12 (ε_{292} = 350 $M^{-1}cm^{-1}$).[35] H_2S was generated by the quantitative reaction between Na_2S and H_2SO_4, and Na_2S concentration corresponded to that of H_2S.[24,28] $^{\bullet}OH$ was quantitatively generated by Fenton reaction between $FeSO_4$ and H_2O_2, and $FeSO_4$ concentrations corresponded to that of $^{\bullet}OH$.[36] Other chemicals were of analytical reagent grade and used without further purification.

Absorption and luminescence analysis

All luminescence measurements were performed using a Fluoro-LS55 Spectrofluorometer with 1.0-cm quartz cells. Absorption spectra were measured on a Cary300 UV–Vis Spectrophotometer with 1.0-cm quartz cells. Compound **1** (3 mL; 10 μM) in 0.1 M PBS (pH 7.4) was added to the cell and was subject to titration with steps of 3 μL of analyte solution. In all titration experiments, the total volume did not exceed 5% of the original volume due to the titration was with steps of 3 μL of different concentration of analyte solution, and each addition of ClO^-/H_2S did not exceed 6 μL. The fluorescent intensity can be kept constant by addition of more concentrated analyte according to the literature procedure.[32] The corresponding luminescence and absorption measurements were taken after the mixture was homogeneously mixed. Commercialized rhodamine B (the quantum yield in ethanol is 0.92) was used as a reference standard,[37] the relative quantum yield of **1** in 0.1 M (pH 7.4) PBS is 0.39 according to the literature method.[38]

MTT assay for the cell cytotoxicity

This involves the reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium to MTT formazan pigment by the metabolic activity of living cells. MCF-7 cells were seeded at a density of 1×10^5 cells/mL in a 96-well plate. After 24 h of cell attachment, cells were treated with **1** (10 μM) for 12 h. Six replicate wells were used for each control and tested concentrations. After incubation for 12 h, the medium was removed and cells were washed with PBS twice. MTT tetrazolium solution (100 μL of 0.5 mg/mL in PBS) was added to each well, and the cells further incubated at 37 ºC for 4 h in a 5% CO_2 humidified atmosphere. Excess MTT tetrazolium solution was then carefully removed and the colored formazan was dissolved in 100 μL dimethyl sulfoxide (DMSO). The plate was shaken for 10 min and the absorbance was measured at 570 and 630 nm using a microplate reader.

In vivo luminescence imaging

All animal experiments were performed in compliance with the Guiding Principles for the Care and Use of Laboratory Animals, Dalian Medical College, China. Healthy mices (7-week-old, 20–25 g) were used and had free access to food and water. All animals were imaged in a latericumbent position using a NightOWL II LB 983 system equipped with an NC 100 CCD deep-cooled camera (Berthold Technologies, Bad Wildbad, Germany), with an excitation filter of 480 nm and an emission filter of 600 ± 20 nm.[39,40] First, **1** (10 μM) in 0.1 M PBS (pH 7.4) was loaded in the leg cortex of mice. Second, 100 μM ClO^- was loaded in the same position. Third, 100 μM H_2S was loaded in the same position. Finally, 100 μM ClO^- was again loaded in the same position.

Results and discussion

The absorption (Fig. S1) and luminescence spectral properties of **1** (10 μM) alone were examined in 0.1 M PBS (pH 7.4) (Fig. 1). The probe showed absorption and luminescence maxima at 450 and 605 nm, respectively. To examine the luminescence response of the probe to ClO^-, **1** was titrated with different concentrations of NaClO in 0.1 M PBS (pH 7.4). As shown in Fig. 1, upon addition of NaClO into the probe solution, the luminescence intensity at 605 nm obviously increased (~7-fold), but the absorption profiles of **1** did not change (Fig. S1).

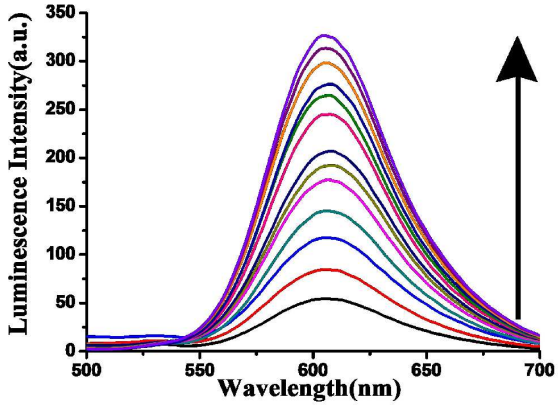


Fig. 1 The emission spectra of **1** (10 μM) upon addition of increasing concentration of ClO^- (0 to 100 μM) in 0.1 M (pH 7.4) PBS, excited at 450 nm ranging 470 nm to 700 nm. The arrow indicated the change of the emission intensities with the increase of ClO^- from 0 to 100 μM.

The luminescence intensity of **1** did not increase when the concentration of ClO^- reached 100 μM (Fig. S2). This was attributed to the formation of a sulfoxide derivative of **1** upon addition of ClO^-, as shown in Scheme 1, and **1** was oxidized into this sulfoxide derivative **2** after ClO^- concentration reached 100 μM. The oxidation product **2** was collected and characterized by mass spectrometry (Fig. S3); the peaks observed at *m/z* 418.6089 (calc. for $[\mathbf{2}\text{-}2PF_6]^{2+}$, 418.6085) and *m/z* 984.1851 (calc. for $[\mathbf{2}\text{-}PF_6]^+$, 984.1853) provide strong evidence for the formation of **2**.

The responses of 10 μM $Ru(bpy)_3^{2+}$ and 10 μM PTZ to ClO^- were investigated in 0.1 M PBS (pH 7.4) as the control experiments. $Ru(bpy)_3^{2+}$ showed a luminescence maximum at 609 nm (Fig. S4), not very different from the luminescence intensity observed upon the addition of NaClO into the $Ru(bpy)_3^{2+}$ solution. PTZ exhibited a

fluorescence maximum at 430 nm (Fig. S5), and the fluorescence intensity increased with increasing concentration of ClO^-, which is in good agreement with the literature.[34] However, only ~0.5-fold increase was observed for PTZ alone, which is much lower compared with that of **1** (~7-fold). These results demonstrate that $Ru(bpy)_3^{2+}$ plays an important role during the abovementioned sensing process.[33]

To examine the influence of pH on the luminescence response of **1** to ClO^-, the system constituted by the probe (10 μM) and ClO^- (100 μM) at different pH values was investigated (Fig. S6). The results demonstrate that the luminescence response of **1** to ClO^- is independent of the pH in the range of 5.0–11.0, thus indicating the good applicability of **1**.

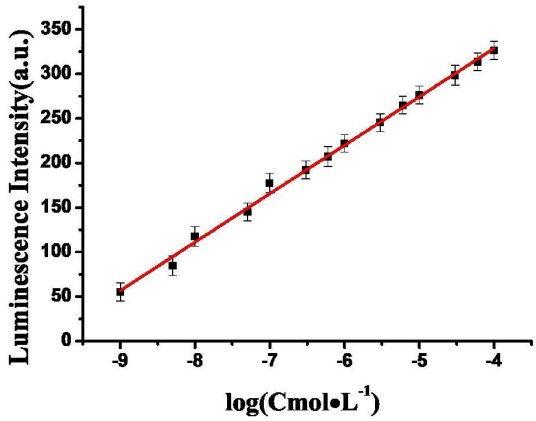


Fig. 2 The linear relationship between the maximum luminescence intensity of 10 μM **1** and the logarithmic concentration of ClO^- (From -9 to -4), the titration was performed in 0.1 M pH 7.4 PBS, with excitation at 450 nm ranging from 470 nm to 700 nm.

A good linear calibration curve (Fig. 2) between the maximum luminescence intensity and the logarithmic concentration of $[ClO^-]$ can be established for ClO^-over the concentration range 1×10^{-9}–1×10^{-4} mol/L, which is much lower than that of other fluorescence probe detection methods.[17-23] The regression equation was $L_{Max} = 546.28 + 54.38 \times \log[ClO^-]$ (Equation 1) with a linear coefficient R = 0.9961; however, the detection limit ($LOD=3S_0/m$)[41,42] decreased to 1.8×10^{-11} mol/L, suggesting that **1** can be suitable in quantitatively detecting ClO^- in aqueous solution.

Table 1 Recovery of ClO^- detected using 10 μM **1** in PBS (0.1M, pH7.4).[a]

No.	Added (mol/L)	Detected (mol/L)	Average (mol/L)	Recovery %	RSD[b] %
1	5.0×10^{-9}	5.10×10^{-9} 5.12×10^{-9} 4.98×10^{-9}	5.06×10^{-9}	101.2	1.50
2	1.0×10^{-7}	0.99×10^{-7} 1.00×10^{-7} 0.97×10^{-7}	0.99×10^{-7}	99.0	1.54
3	5.0×10^{-6}	5.10×10^{-6} 5.13×10^{-6} 5.11×10^{-6}	5.11×10^{-6}	102.2	0.31
4	1.0×10^{-4}	1.10×10^{-4} 1.11×10^{-4} 1.13×10^{-4}	1.11×10^{-4}	111.0	1.40

[a] Average of three determinations and the averaged readings were used. [b] RSD stands for relative standard deviation.

To assess the accuracy of the proposed method, 10 μM **1** was used to determine ClO^- in 0.1 M PBS (pH 7.4). Four tap water samples with different concentrations of ClO^- were studied. The sample solution (3 μL) was added into 3 mL of 10 μM **1** in 0.1 M PBS, the luminescence was measured, and the concentration of ClO^- was calculated according to Equation 1. The results are shown in Table 1. The relative standard deviation was less than 1.54% for ClO^-, indicating the fine accuracy of the method and further demonstrating its applicability.

To evaluate the selectivity of **1** toward ClO^-, the probe was treated with various cations (Ca^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+}, Mg^{2+}, Al^{3+},and Fe^{3+}), anions (CH_3COO^-, I^-, SO_4^{2-}, ClO_3^-, SO_3^{2-}, NO_3^-, NO_2^-, and ClO^-), and other ROS such as $^{\bullet}OH$, Na_2MoO_4, H_2O_2, KO_2, and peroxynitrite ($ONOO^-$). The luminescence intensity changes of **1** (10 μM) with various substances within 30 min in 0.1 M PBS (pH 7.4) are shown in Fig. S7. Almost no changes in the luminescence intensity of **1** can be observed upon the addition of the abovementioned analytes except for ClO^-, demonstrating that **1** is selective to ClO^-.

To investigate the reversible luminescence response of **1** to the ClO^-/H_2S redox cycle in aqueous solution, 10 μM **1** in 0.1 M PBS (pH 7.4) was successively titrated with 100 μM ClO^- and different concentrations of H_2S. After the addition of 100 μM ClO^- into the probe solution, the luminescence intensity of **1** reached a maximum constant value in approximately 10 min. Then, the luminescence intensity of the solution gradually decreased and returned to the original level upon the addition of different concentration of H_2S (Fig. 3 and Fig. S8). As shown in Scheme 1, the oxidation derivative **2** (sulfoxide form of **1**) could be restored to **1** by adding the same equivalents of H_2S (3 μL, 100 μM).

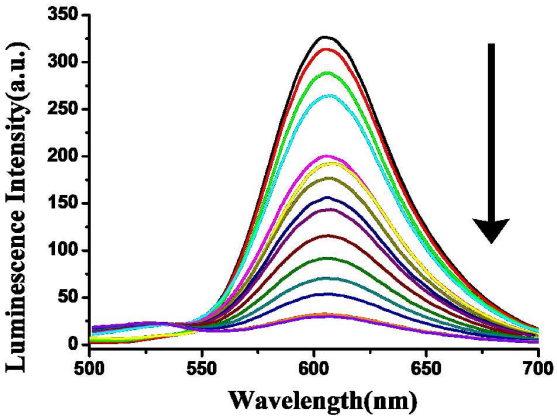


Fig. 3 Emission spectra of the 10 μM oxidation form of **1** upon the addition of increasing concentration of H_2S (0 to 100 μM) in 0.1 M (pH 7.4) PBS excited at 450 nm ranging 470 nm to 700 nm. The arrow indicated the change of the emission intensities with the increase of H_2S from 0 to 100 μM.

A good linearity between the maximum luminescence intensity and $\log[H_2S]$ was also observed (Fig. S9). The regression equation was $L_{max} = -231.9194 - 62.6113 \times \log[H_2S]$ (Equation 2) with a linear coefficient R = 0.993. The detection limit ($LOD = 3\ S_0/m$)[41,42] was 1.2×10^{-11} mol/L for H_2S, which is much lower than that of other fluorescence detection methods.[24-31]

To assess the accuracy of the proposed method, **2**, the oxidation form of **1** (10 μM) was used to determine H_2S in 0.1 M PBS (pH 7.4). Four tap water samples with different concentrations of H_2S were studied. The sample solution (3 μL) was added to 3 mL of 10 μM **1** and 100 μM ClO^- in 0.1 M PBS (pH 7.4), the luminescence was measured, and the concentration of H_2S was calculated

according to Equation 2. The analytical results are shown in Table S1. The relative standard deviation was less than 5.80%, showing the fine accuracy of the method in the luminescence measurement of H_2S and further demonstrating its applicability.

The selectivity of the sulfoxide derivative of **1** to different reductants ($NaHSO_3$, Hcys (homocysteine), CyS (cysteine), GSH (glutathione), VC (vitmit C), H_2S) was also investigated within 30 min (Fig. S10). The results showed that H_2S could quickly recover the luminescence intensity in 5 min, but the others could not. Therefore, **1** was chosen as a rapid, highly selective luminescent probe for monitoring the redox cycle between ClO^- and H_2S in aqueous solution. As shown in Fig. 4, the redox cycle can be repeated at least 12 times with good reproducibility and stability. The volume of the probe solution did not vary much because each addition of ClO^-/H_2S did not exceed 6 μL. The total volume added was 78 μL.

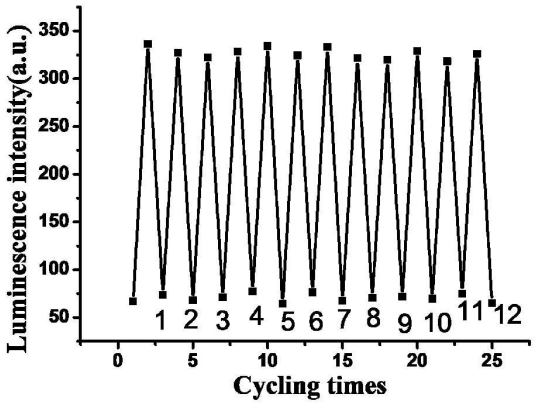


Fig. 4 Luminescence response of **1** (10 μM) to $HClO/H_2S$ redox cycles. **1** was first oxidized by ClO^-, 10 min later; the solution was treated with H_2S. When the luminescence returned to original levels, another portion of ClO^- was added. The redox cycles were repeated at least twelve times. All spectra were measured in 0.1 M PBS (pH 7.4), λ_{ex} = 450 nm and λ_{em} = 605 nm. The numbers in figure represented the redox cycles times. The titrated concentrations of ClO^- and H_2S were: 1, 100 μM (3 μL, 0.1 M) ClO^- and 100 μM (3 μL, 0.1 M) H_2S; 2, 300 μM (3 μL, 0.2 M) $ClO^-$and 200 μM (3 μL, 0.1 M) H_2S; 3, 500 μM (3 μL, 0.2 M) ClO^- and 300 μM (3 μL, 0.1 M) H_2S; 4, 700 μM (3 μL, 0.2 M) ClO^- and 500 μM (3 μL, 0.2 M) H_2S; 5, 1 mM (3 μL, 0.3 M) ClO^- and 800 μM (3 μL, 0.3 M) H_2S; 6, 4 mM (3 μL, 1 M) ClO^- and 1 mM (3 μL, 0.2 M) H_2S; 7, 6 mM (3 μL, 2 M) ClO^- and 3 mM (3 μL, 2 M) H_2S; 8, 9 mM (3 μL, 3 M) ClO^- and 5 mM (3 μL, 2 M) H_2S; 9, 20 mM ClO^- (3 μL, 10 M) and 7 mM (3 μL, 2 M) H_2S; 10, 40 mM ClO^- (6 μL, 10 M) and 10 mM (3 μL, 3M) H_2S; 11, 60 mM (6 μL, 10 M) ClO^- and 13 mM (3 μL, 3 M) H_2S; 12, 70 mM (3 μL, 10 M) ClO^- and 15 mM (3 μL, 2 M) H_2S.

According to the literature procedure,[43] the MTT assay in the MCF-7 cell lines are carried out to confirm the cytotoxicity of **1**. Figure 5 shows that **1** has low toxicity in vivo for 12 h.

Finally, **1** was used to visualize the redox cycle induced by ClO^- and H_2S in live mice. The mice loaded with 10 μM **1** exhibited weak luminescence (Fig. 6a). An intense luminescence was produced by stimulating the probe-loaded mice with 100 μM ClO^- (Fig. 6b), due to the oxidation of **1** to **2** by ClO^-. After H_2S was injected into the mice, the luminescence returned to its original state (Fig. 6c) because H_2S reduced **2** to **1**. Then, the luminescence intensity was intensively enhanced again after another 3 μL of 100 μM ClO^- was injected into the mice (Fig. 6d). These results clearly highlight the ClO^-/H_2S redox cycle in response to **1**. To study the inhibitor effect on the selectivity of **1** for the redox cycle between ClO^- and H_2S, PABA (Para aminobenzoic acid, Hypochlorous acid inhibitor)[44] was employed in the control experiments. The results are shown in Figure S11. First, **1** (10 μM) in 0.1 M PBS (pH 7.4) was loaded in the leg cortex of the mice. Second, 600 μM PABA was loaded in the same positioin. Third, 100 μM ClO^- was loaded in the same position. Fourth, another 100 μM ClO^- was loaded in the same position. Finally, 100 μM H_2S was loaded in the same position. Attributed to the inhibiting effect of PABA for ClO^-, no response of **1** for ClO^- can be observed when PABA was loaded on mice, providing further evidence that **1** has high selectivity for the redox cycle ClO^- and H_2S in vivo.

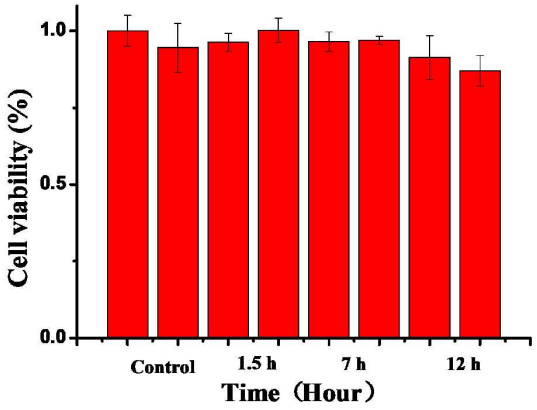


Fig. 5 Relative cell viability of MCF-7 in 0.1 M PBS (pH 7.4) with **1** (10 μM) after 12 h of incubation determined using the MTT assay by monitoring formazan absorbance at 480 nm; Mean values and standard deviation from five independent experimental determinations.

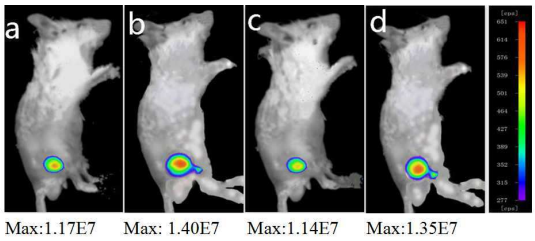


Fig. 6 The luminescence imaging of the redox cycle between ClO^- and H_2S in live mice. a) **1** (10 μM, 100 μL) in 0.1 M PBS (pH 7.4) was loaded in the leg cortex of the mice. b) 100 μM ClO^- (0.1 μL) was loaded in the same position. c) 100 μM H_2S (0.1 μL) was loaded in the same position. d) another 100 μM ClO^- (0.1 μL) was loaded in the same position.

Conclusions

In conclusion, a reversible and highly selective luminescent probe (**1**) was successfully employed for the determination of ClO^- and H_2S and for monitoring the ClO^-/H_2S redox cycle both in vitro and in vivo. The luminescent probe (**1**) can sensitively and selectively detect the ClO^-/H_2S redox cycle in vitro and in vivo. The redox cycle response of the probe (**1**) for ClO^-/H_2S can be repeated at least 12 times with good reproducibility and stability. These results provide evidence for potentially monitoring the ClO^-/H_2S redox cycle by using a luminescent method.

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Notes and references

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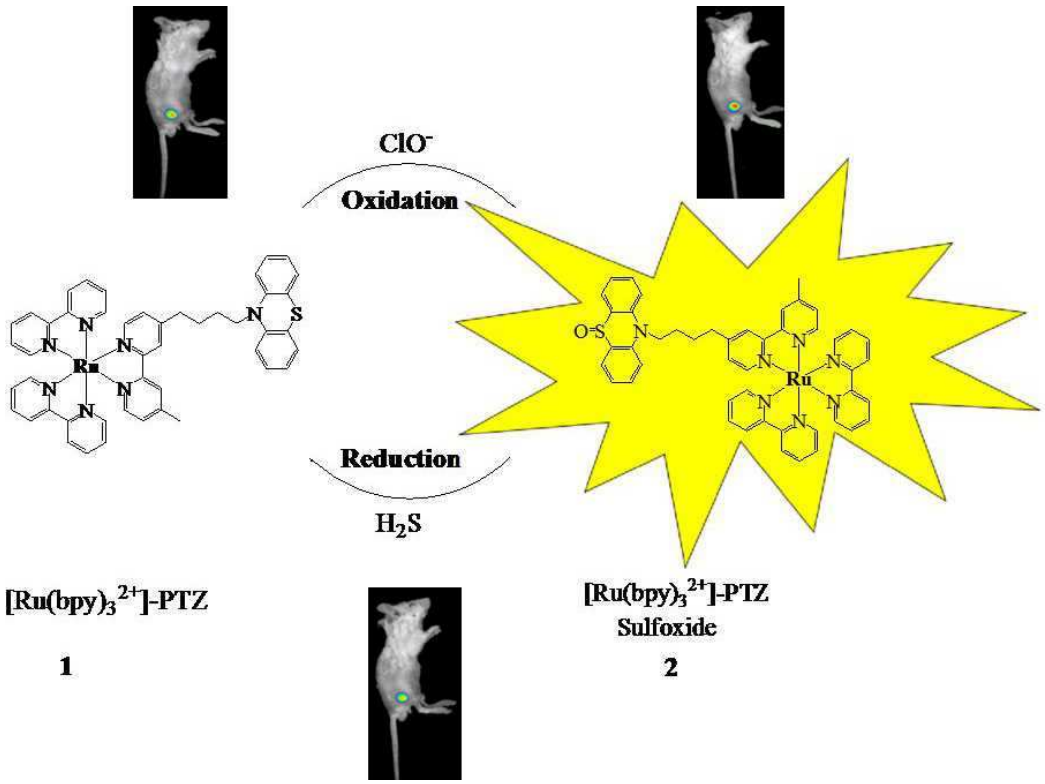
Graphic Abstract



A reversible and highly selective luminescent probe was successfully employed for the determination of ClO^- and H_2S and for monitoring the ClO^-/H_2S redox cycle both in vitro and in vivo.