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RAPID COMMUNICATION

Fluorescence and HPLC Detection of Hydroxyl Radical by a Rhodamine-Nitroxide Probe and its Application in Cell Imaging

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Abstract A rhodamine nitroxide probe was designed to detect the hydroxyl radical (·OH), which presented high selectivity for ·OH over other reactive oxygen species (ROS) and linear fluorescence response to ·OH produced by Fenton reaction. The product was detected by HPLC-MS, indicating that the main product of the reaction was O-methylhydroxylamine and the product peak areas measured by HPLC-UV/vis and HPLC-FLD both enhanced proportionally with the increase of ·OH concentration. The application of the probe in biological system was explored to trace the production of ·OH in cells under oxidative stress condition induced by rotenone which can inhibit the mitochondria respiratory chain complex I and we found that appropriate rotenone may induce the normal human liver cells (L02) and human hepatoma cells (HepG2) to produce ·OH at different degrees.

Keywords Hydroxyl radical · Fluorescence · Nitroxide · Oxidative stress · Spin label

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Introduction

The excess production of reactive oxygen species (ROS) has the potential to cause cellular damage and may aid in initiating radical chain reactions [1,2], such an imbalance of the cellular redox environment is describe as oxidative stress. Among the various ROS, the hydroxyl radical (·OH) is most aggressive which can cause DNA damage and result in gene mutation [3].

Hence, many methods have been developed for the detection of ·OH [4], such as electron spin resonance (ESR) [5,6] and aromatic hydroxylation [7,8]. But the characterizations such as insensitive, low selectivity, complex products and inconvenience for quantitative detection limit their use. In addition, some new ratiometric probes based on the ·OH induced cleavage of a DNA strand was developed for the detection of ·OH [9,10], but the probes suffer from complexity of the synthesis process and the FRET mechanism (fluorescence resonant energy transfer) limit the use of dyes for the requirement of the energy matching between the two fluorophores. Recently, the spin labeled fluorescent probes (F-NO·) have been used to detect the ·OH actively for the high sensitive, selective, uncomplicated product and more importantly the flexible choose of the fluorophores [11–14]. Because the fluorescence of the fluorophore can be quenched by the nitroxide efficiently through electron exchanges but recovered after conjugating with a free radical or by reduction [15], the probes are usually called profluorescent probe. F-NO· probes with long wavelength have also been used for fluorescence imaging of the cellular oxidative stress [16–18]. Despite the active use of the F-NO· probes, far less attention has been given to the quantitative detection of the ·OH.

Rhodamine, as a classic dye, has been used in biological imaging widely for its excellent photophysical and photochemical properties [19–21]. In the present manuscript, a spin labeled fluorescence probe based on rhodamine B was developed to detect the ·OH in vitro and in the cells under oxidative

stress condition induced by rotenone (an inhibitor of the mitochondrial respiratory chain complex I). As mitochondria are a major source of intracellular ROS and the impairment of complex I caused by rotenone will result in the production of the ROS and free radicals [2,22–24]. For the product is simply, efforts was also paid to the quantitative detection of ·OH by HPLC. The structure of the probe and the detection mechanism were shown in Scheme 1.

Experimental Section

Materials and Methods

4-hydroxy-2, 2, 6, 6-tetramethyl-piperidinooxy free radical (4-OH-TEMPO), Rhodamine B, rotenone were purchased from Sigma-Aldrich. HepG2 and L02 cells were purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. All reagents used were of analytical grade or chromatographically pure.

ESI-MS studies were carried out with a Esquire 5000 spectrometer and NMR spectra were performed on a Varian INOVA 400 MHz spectrometer. HPLC-MS were recorded out on an Agilent 1100 series LC/MSO. The fluorescence spectra were recorded on a Perkin Elmer LS55 spectrometer while the fluorescence and bright field images were acquired with Olympus BX51.

Synthesis of the Probe

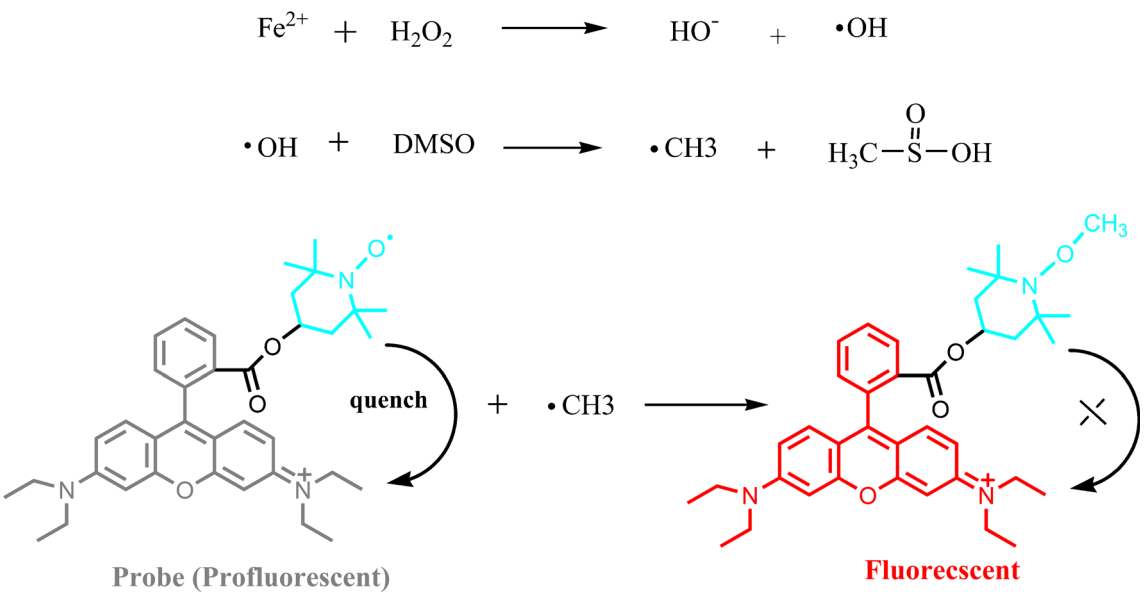
Rhodamine B (0.27 mmol) was mixed with equimolar 4-OH-TEMPO in dichloromethane, and then 4-dimethylamiopryidine (DMAP, 0.027 mmol) and dicyclohexyl carbodiimide (DCC, 0.27 mmol) were added as catalyst and dehydrating agent. The mixture was stirred under Argon atmosphere for 24 h at ambient. After removing the solvent under vacuum, the residue was purified by chromatography on neutral aluminum oxide column (eluent: CH_2Cl_2/CH_3OH 20/1, v/v). The probe was obtained in 31 % yield and stored in a −20 °C freezer. ^1H NMR (400 MHz, $CDCl_3$, tetramethylsilane (TMS)): δ (ppm)=6.7–8.2 (aryl H, 11 H), 3.4–3.7 (NCH_2; OCH, 9 H), 1.2–1.7 (CH_3; CH_2, 24 H); MS (ESI-MS): m/z calcd: 597.36, found: 597.5 $[M]^+$(The synthetic route of the probe was shown in scheme S1, supplementary information).

HPLC-MS Analysis of the Reaction Product

The reaction mixture of the probe and Fenton system was stirred in CH_3CN for 30 min at ambient, and then subjected to the HPLC analysis with the conditions: eluent, CH_3OH (85 %)/ammonium acetate (0.01 M, 15 %) aqueous solution; flow rate, 1.0 ml/min; injection volume, 20 μl; temperature, 25 °C; analytical column, ctadecyl (C18). The elution was monitored by HPLC-UV/Vis-MS.

Fluorescence Imaging

HepG2 and L02 cells were cultured in MEM medium supplemented with 10 % fetal calf serum and incubated at 37 °C in 5 % CO_2/air atmosphere. Cells were seeded into culture dishes with appropriate density and cultured for 12 h, then choose the well-grown cells and treated accordingly. The probe was added to all cell samples at a final concentration of 1 μM. A fresh stock solution of rotenone (0.1 mM) was prepared in DMSO and appropriate concentration was added to the cell samples.



Scheme 1 The detection mechanism of the ·OH produced by Fenton reaction

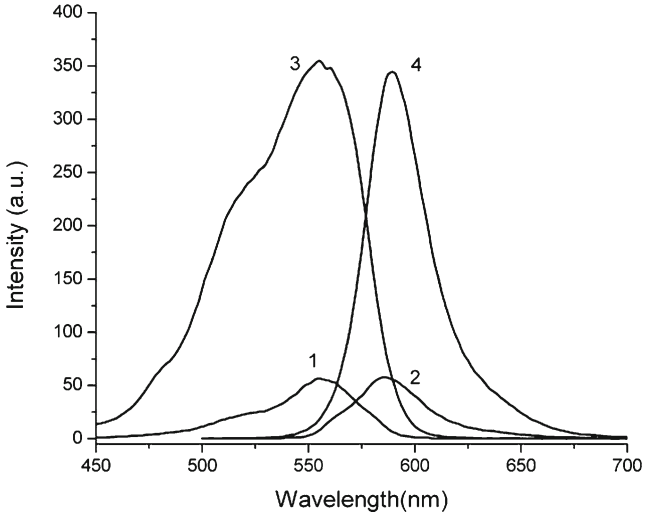


Fig. 1 The excitation (lines 1 and 3) and emission spectra (lines 2 and 4) of the probe (7.5 μM) react with (3, 4) or without (1, 2) Fenton reagent ([Fe^{2+}]/H_2O_2, mol/mol=1:6) for 30 min. Final [Fe^{2+}]:100 μM, DMSO: 1 %

The cells were treated with rotenone for 30 min before incubated with 1 μM probe for another 2 h. After all the treatment, the medium was removed from the cells and washed 3 times with 10 % PBS. Fluorescence and bright field images were acquired with Olympus BX51 with 10× eye lens and 20× objective lens.

Results and Disussion

The spectral changes of the probe were tested by its reaction with ·OH produced by Fenton reaction. As the Fig. 1 shows, the probe represented weak fluorescence before reaction indicating that the nitroxide can quench the fluorescence of rhodamine B efficiently. But the fluorescence recovered significantly after reacting with ·OH and the λ_{max} of the excitation and emission were 556 nm and 588 nm. At the same time, the fluorescence intensity of the probe enhanced with the increase of the ·OH concentration and the relative fluorescence intensity ($F-F_0$) was linear response to the concentrations of Fe^{2+} which represent the ·OH concentrations (Fig. 2).

The reaction product was detected by HPLC-MS. Compared with previous HPLC detection method such as the aromatic hydroxylation of the terephthalic acid [7,25], salicylic acid [8] and phenylalanine [26] by reacting with ·OH directly, which were complicated to detect quantitatively for the multiple products, the product of the present method is simply with only one main product as can be seen from Fig. 3. The peak a (probe, 14.4 min) and peak b (product O-methylhydroxylamine, 16.5 min) were determined by HPLC-MS (the MS spectra were shown in Fig. S1 and S2, supplementary information). The total fluorescence response was very weak before reaction but increased significantly after reacting with ·OH (Fig. 3b). At the same time, the product peak b became stronger with the increase of ·OH concentration but the peak a (probe) became weaker and almost disappeared at last, which can also be seen from the HPLC-UV/vis chromatogram obviously (Fig. 3a). In addition, it is clear that the peak a (probe) with strong absorbance presented very weak fluorescence, which also indicated that the nitroxide can quench the fluorescence of rhodamine B efficiently.

More importantly, the integral area of product peak b detected by UV/vis and FLD both enhanced proportional to the ·OH concentration (Fig. 4). At the same time, the correlation between the signal integral area of product peak b measured by UV/vis and FLD was very good (R=0.9989). The results above denoted that the probe is appropriate as a pre-column reagent to detect the ·OH quantitatively by HPLC (The HPLC

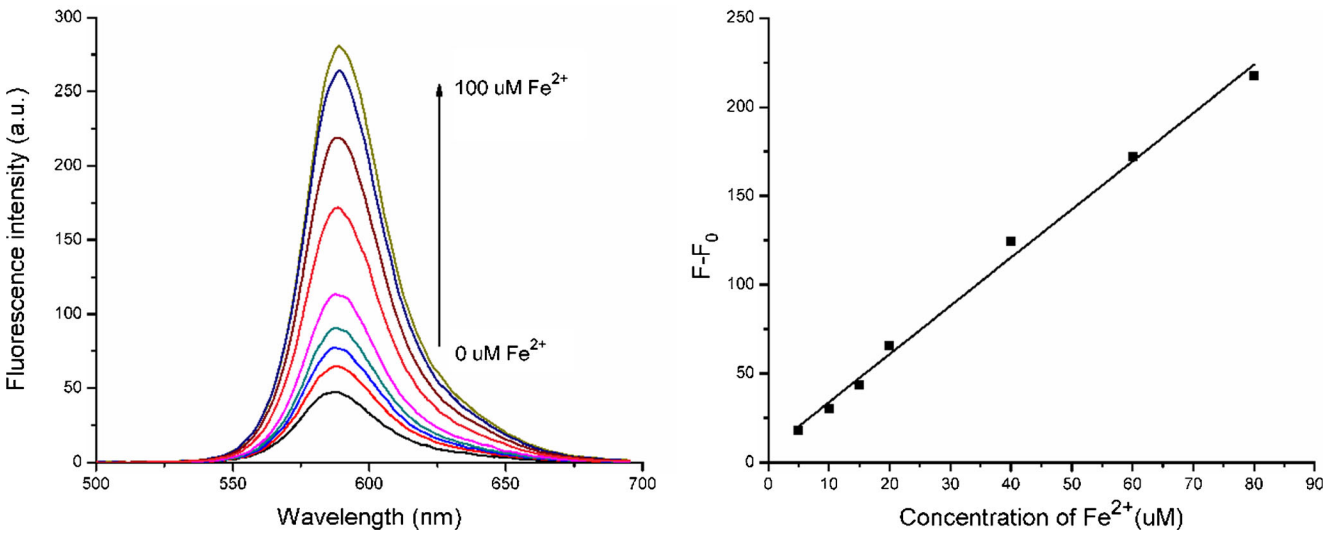


Fig. 2 Fluorescence responses of the probe (5 μM) toward various concentrations of Fenton reagent ([Fe^{2+}]/H_2O_2, mol/mol=1:6). Final [Fe^{2+}]: 0–100 μM, DMSO 1 %. $F-F_0$=6.70 +2.71[Fe^{2+}], R^2=0.9935

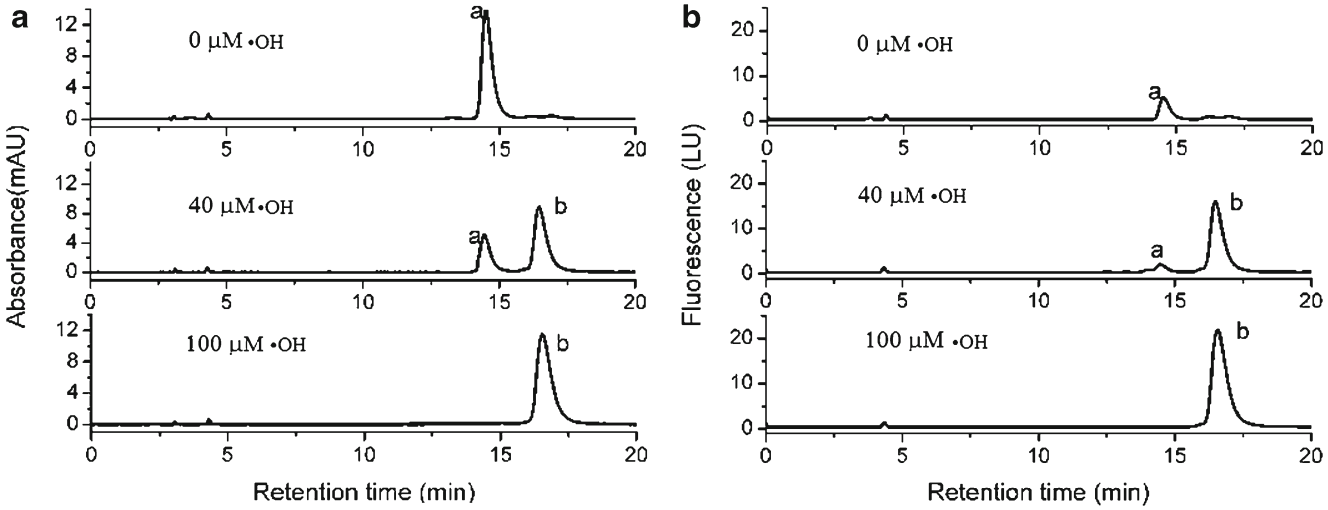


Fig. 3 HPLC detection of reacting product (**a** was HPLC-UV/vis detection; **b** was HPLC-FLD detection). Probe (10 μM) reacted with 0 μM, 40 μM and 100 μM ·OH in CH_3CN containing 1 % DMSO for 30 min. ·OH was produced by Fenton reagent ($[Fe^{2+}]/H_2O_2$, mol/mol=1:6), final $[Fe^{2+}]$ represented the [·OH]. (a peak retention time, 14.4 min; b peak retention time 16.5 min; FLD, Ex 556 nm, Em 590 nm; UV/vis, absorbance at 550 nm)

chromatograms of the probe response to various ·OH were given in Fig. S3 and S4, supplementary information).

The interference of other ROS was tested. As Fig. 5 shows the probe showed highly selectivity for ·OH over other ROS investigated, such as hydrogen peroxide (H_2O_2), hypochlorite (OCl^-) and peroxynitrite ($ONOO^-$). In addition, the ·OH alone also had little effect on the fluorescence of the probe, indicating that the DMSO is necessary for the fluorescence recovery as the mechanism shown in Scheme 1.

The influence of DMSO which was used as the ·OH scavenger on the cell vitality was studied by cytometry. The population of the HepG2 cells treated with DMSO lower than 0.5 % did not show obvious changes compared with the blank (Fig. S5, supplementary information). So in order to scavenge the ·OH as full as possible, 0.5 % DMSO was used in the following cellular experiment.

To expand the use of the probe in biological systems, we applied the probe to explore the oxidative stress of the HepG2 and L02 cells induced by rotenone (an inhibitor of mitochondria respiratory chain complex I). It is clear that the fluorescence of HepG2 cells treated with rotenone (Fig. 6b) was stronger than the blank (Fig. 6a) obviously. Because mitochondria are a major source of intracellular ROS and the impairment of complex I caused by rotenone will result in the production of the ROS and the free radicals [2,22–24], the probe can conjugate with the methyl radical

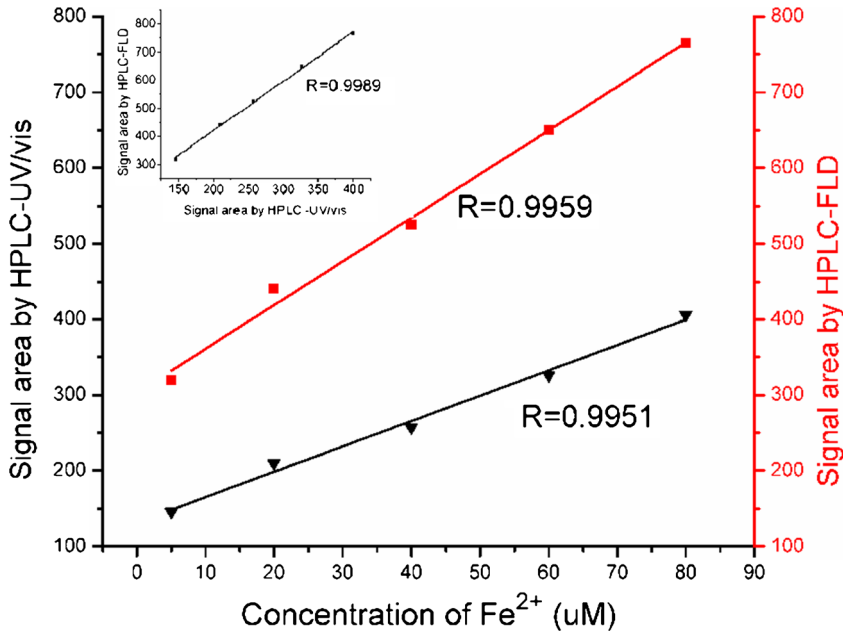


Fig. 4 The linear relationship between signal integral area of product peak b detected by HPLC and the ·OH concentration (Blank was HPLC-UV/vis detection; red was HPLC-FLD detection). Probe (10 μM) reacted with 0–80 μM ·OH in CH_3CN containing 1 % DMSO for 30 min. ·OH was produced by Fenton reagent ($[Fe^{2+}]/H_2O_2$, mol/mol=1:6), final $[Fe^{2+}]$ represented the [·OH]. The inset is the linear relationship between the signal integral area of product peak b measured by UV/vis and FLD

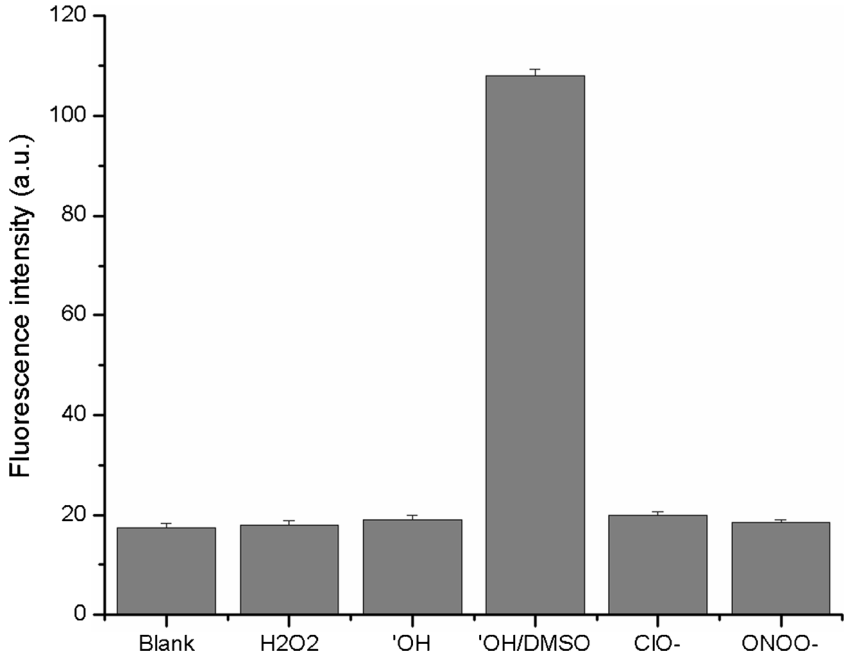


Fig. 5 Fluorescence response of the probe (2 μM) toward various ROS (5 μM). ·OH was produced by Fenton reaction ([Fe^{2+}] 5 μM, [Fe^{2+}]/H_2O_2, mol/mol=1:6); $ONOO^-$ was prepared by KNO_2 and H_2O_2 in HCl according to a previous report [27]. DMSO added was 1 %. The error bars represent the standard deviation of three measurements conducted for each ROS

produced by the reaction of ·OH with DMSO and lead to the fluorescence increase. The same phenomenon was observed for L02 cells (Fig. 6c and d) except that the fluorescence increase of the HepG2 cells was more significantly than L02 cells, which indicated that the rotenone may induce the HepG2 cells to produce more ·OH than L02 cells. In addition, the cell morphology of the cells treated with the probe and rotenone presented no obvious changes, implying that the probe and rotenone had low cytotoxicity on cells under these experimental conditions. The results above foreshadow broad application prospects of the probe in biological systems.

In summary, the method we proposed to detect the ·OH is sensitive, selective and convenient to detect quantitatively by HPLC for the simple product compared with the previous methods of aromatic hydroxylation. The probe has the potential to trace the ·OH production under oxidative stress conditions induced by the impairment of the mitochondrial respiratory chain, such as the oxidative stress induced by rotenone (an inhibitor of complex I) which has been explored in the present manuscript. More applications will be explored in biological system. Especially the extension of this method to detect ·OH resulting from radiation is under active research in our laboratory.

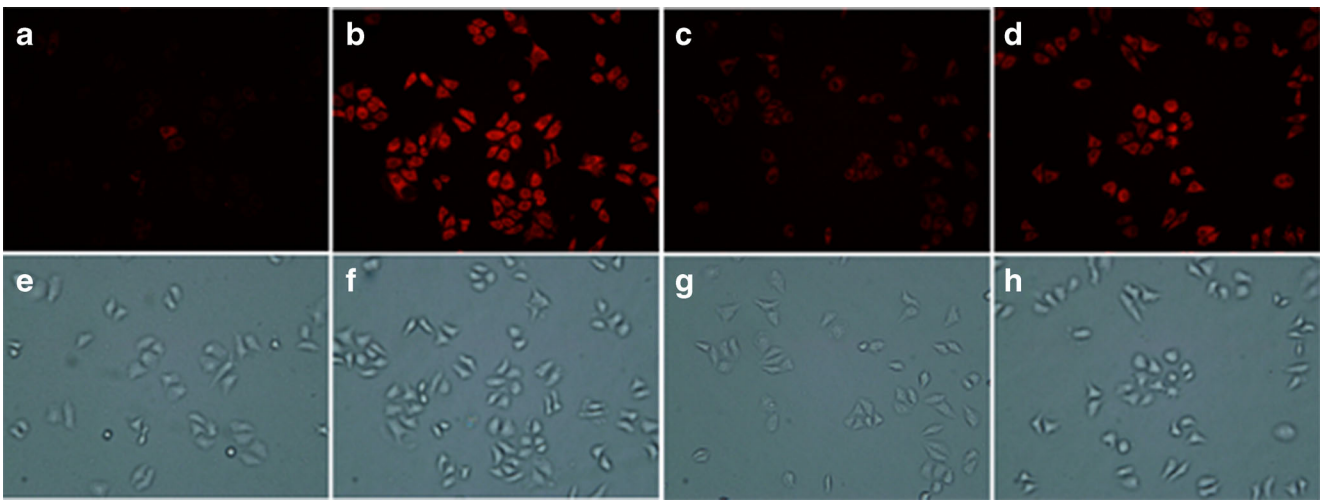


Fig. 6 Cell imaging of ·OH production induced by rotenone. **a** HepG2 cells incubated with 1 μM probe for 2 h, **b** HepG2 cells treated with 1 μM rotenone for 30 min before incubated with 1 μM probe for another 2 h, (**c**) and (**d**) were L02 cells treated the same as (**a**) and (**b**) respectively, **e**, **f**, **g**, **h** were the corresponding bright field images (all the cell samples were added 0.5 % DMSO before added the probe)

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