A highly selective fluorescent probe for hydrogen peroxide and its applications in living cells

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Abstract

A turn-on fluorescent probe **ACR**{6'-[2-(azidomethyl)benzoyl]-7'-chloro-*N,N*-diethylrhodol} for selective detection of H₂O₂ was developed. An ester bond was broken by the nucleophilic substitution of H₂O₂ which released strong fluorescence. Probe **ACR** was proved to be made up of three essential parts, chlorine atom, azide group and an active carbonyl. This probe showed high selectivity for H₂O₂ and a linear fluorescence intensity enhancement with a wide range of concentrations of H₂O₂. Fluorescence imaging experiments in HeLa cells indicated its potential to image H₂O₂ in biological systems.

Keywords

Hydrogen peroxide, Selective detection, Fluorescent probe

1. Introduction

Hydrogen peroxide (H_2O_2), a precursor molecule of other reactive oxygen species (ROS) [1], plays a crucial role in many biological processes in the human body, such as respiration[2], intracellular signaling[3], and immune responses[4-5]. However, over production of H_2O_2 may cause many diseases, such as neurodegenerative diseases [6], inflammatory diseases [7], Alzheimer's diseases [8] and cancers[9]. Therefore, it is necessary to develop sensitive and selective methods for the detection of H_2O_2 in living systems.

Currently, many chemical tools have been developed to detect intracellular H_2O_2 , such as mass probes[10], proteomics probes[11], and fluorescent probes[12,13]. Among these methods, fluorescence methods have gained a lot of attention due to their fast response, high sensitivity, and ability to afford high spatial resolution through microscopic imaging[14]. In consequence, various fluorescent probes have been developed and applied for H_2O_2 analysis for the past few years[12-13,15-31]. Meanwhile, several unique H_2O_2 -responsive sites were developed, such as boronate [12,15-18], arylsulfonyl esters [19,20], diphenylphosphine [21], α -diketone groups [22-25], metal complexes [26-29] and chalcogen [30,31]. Although some of these probes have permitted the detection and quantification of intracellular H_2O_2 , such probe systems lack full applicability to biological systems due to their modest selectivity, requirements for external enzymes or incompatibility with biological milieus [28]. So it is especially important to develop high-selectivity probes to monitor the H_2O_2 in the biological process.

2. Experimental

2.1. Materials and instruments

Fluorescence spectra were carried out on an Edinburgh Instruments Ltd-FLS920 fluorescence

spectrophotometer. UV/vis spectra were made with a Shimadzu UV-2600 spectrophotometer. All reagents and solvents were purchased from commercial suppliers and used without further purification unless otherwise stated. Deionized water was used throughout all experiments. All reactions were magnetically stirred and monitored by thin layer chromatography (TLC). Column chromatography was performed using 200-300 mesh silica gel supplied by Qingdao Marine Chemical Factor. The ^1H NMR and ^{13}C NMR spectra were recorded on a Bruker AVANCE 400 at 400 and 100 MHz, respectively. All NMR chemical shifts were referenced to residual solvent peaks or to $\text{Si}(\text{CH}_3)_4$ as an internal standard. NMR spectra were recorded in CDCl_3 or $\text{DMSO}-d_6$. Chemical shifts were referenced to residual CHCl_3 at 7.26 ppm for ^1H or 77.0 ppm for ^{13}C . All coupling constants J are quoted in Hz. FTIR spectra were obtained with a Bruker Vertex 70 FT-IR spectrometer with KBr pellets. All IR samples were prepared as thin film and reported in wave numbers (cm^{-1}). High resolution mass spectra were obtained on a Q-TOF6510 instrument mass spectrometer.

2.2. Synthesis

Synthesis of 7'-chloro-*N,N*-diethylrhodol

To a suspension of 2-(4-diethylamino-2-hydroxybenzoyl)benzoic acid (6.0 g, 19.1 mmol) and resorcinol (2.1 g, 19.1 mmol) in trifluoroacetic acid (TFA, 20 mL) in a sealing tube, was added molecular sieve (2.0 g). The resulting mixture was heated to 90 °C and stirred for 6 h. Then the mixture was cooled to room temperature and concentrated to yield a crude product, which was

recrystallized in a mixture of ethyl acetate and petroleum (V:V = 1:1) affording 7'-chloro-*N,N*-diethylrhodol as a red solid (6.8 g, 84.5% yield). Data for 7'-chloro-*N,N*-diethylrhodol: R_f 0.35 (petroleum ether:ethyl acetate = 1:1); ^1H NMR (400 MHz, $\text{DMSO-}d_6$) δ 8.07 (d, J = 6.8 Hz, 1H), 7.56–7.58 (m, 2H), 7.17 (dd, J = 6.8, 1.6 Hz, 1H), 6.86 (s, 1H), 6.77 (d, J = 9.6 Hz, 1H), 6.71 (d, J = 6.8 Hz, 1H), 6.70 (s, 1H), 6.36 (s, 1H), 3.47 (q, J = 6.8 Hz, 4H), 1.13 (t, J = 6.8 Hz, 6H); HRMS-ESI (m/z) $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{24}\text{H}_{21}\text{ClNO}_4^+$, 422.1154, found: 422.1151; mp 177.9–179.5°C.

Synthesis of **ACR**

A solution of 2-(azidomethyl)benzoic acid (252.2 mg, 1.4 mmol) in DCM (5 mL), was added 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI, 341.5 mg, 1.8 mmol), 4-dimethylaminopyridine (DMAP, 72.5 mg, 0.59 mmol) and 7'-chloro-*N,N*-diethylrhodol (500.0 mg, 1.19 mmol). The resulting mixture was warmed to room temperature and stirred for 12 h. The resulting solution was poured into water (30 mL) and extracted with DCM (3×20 mL). Combined organic layers were washed with brine, dried over anhydrous MgSO_4 and concentrated to afford red oil. Flash chromatography of the crude product (5:1 petroleum ether:ethyl acetate) provided the desired product as a colorless oil (358.81 mg, 52%). Data for **ACR**: R_f 0.35 (petroleum ether:ethyl acetate = 5:1); ^1H NMR (400 MHz, CDCl_3) δ 8.36 (dt, J = 9.2, 4.6 Hz, 1H), 8.07 (d, J = 7.5 Hz, 1H), 7.74 (tt, J = 4.1, 2.0 Hz, 1H), 7.71–7.69 (m, 1H), 7.69–7.65 (m, 1H), 7.63 (d, J = 7.2 Hz, 1H), 7.60–7.52 (m, 1H), 7.28 (d, J = 3.4 Hz, 2H), 6.90 (s, 1H), 6.60 (d, J = 8.9 Hz, 1H), 6.47 (d, J = 2.5 Hz, 1H), 6.41 (dd, J = 9.0, 2.6 Hz, 1H), 4.99–4.75 (m, 2H), 3.46–3.18 (m, 3H), 1.24–1.10 (m, 6H); ^{13}C NMR (100 MHz, CDCl_3) δ 169.25, 163.75, 152.55, 150.97, 149.80, 147.87, 138.68, 135.14, 133.96, 132.00, 129.93, 129.90, 129.21, 128.82,

128.41, 126.84, 126.79, 125.15, 124.16, 121.33, 119.04, 112.67, 108.86, 104.33, 97.57, 83.01, 52.98, 44.52, 12.49; IR (thin film) ν_{max} 3450, 2918, 2113, 1751, 1419, 1343, 1143, 1049, 811, 745; HRMS-ESI (m/z) $[M + H]^+$ calcd for $C_{32}H_{26}ClN_4O_5^+$, 581.1513, found: 581.1523.

Synthesis of **MCR**

A solution of 2-methylbenzoic acid (194.6 mg, 1.43 mmol) in DCM (5 mL), was added EDCI (341.5 mg, 1.78 mmol), DMAP (72.5 mg, 0.59 mmol) and 7'-chloro-*N,N*-diethylrhodol (500.0 mg, 1.19 mmol). The resulting mixture was warmed to room temperature and stirred for 12 h. The resulting solution was poured into water (30 mL) and extracted with DCM (3×20 mL). Combined organic layers were washed with brine, dried over anhydrous $MgSO_4$ and concentrated to afford orange oil. Flash chromatography of the crude product (5:1 petroleum ether:ethyl acetate) provided the desired product as a colorless oil (250.0 mg, 39%). Data for **MCR**: R_f 0.35 (petroleum ether:ethyl acetate = 5:1); 1H NMR (400 MHz, $CDCl_3$) δ 8.24 (d, $J = 7.9$ Hz, 1H), 8.05 – 8.02 (m, 1H), 7.71 (td, $J = 7.5, 1.2$ Hz, 1H), 7.65 (dd, $J = 7.5, 1.0$ Hz, 1H), 7.50 (dd, $J = 7.5, 1.3$ Hz, 1H), 7.35 (t, $J = 8.0$ Hz, 2H), 7.24 (d, $J = 6.7$ Hz, 2H), 6.87 (s, 1H), 6.57 (d, $J = 8.9$ Hz, 1H), 6.45 (d, $J = 2.5$ Hz, 1H), 6.38 (dd, $J = 9.0, 2.6$ Hz, 1H), 3.36 (q, $J = 7.0$ Hz, 4H), 2.68 (s, 3H), 1.18 (t, $J = 7.0$ Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.23, 164.30, 152.54, 152.49, 150.89, 149.73, 148.20, 141.77, 135.06, 133.20, 131.99, 131.47, 129.86, 129.08, 128.77, 127.41, 126.87, 126.02, 125.07, 124.12, 121.48, 118.69, 112.70, 108.76, 104.34, 97.52, 83.03, 44.47, 21.84, 12.44; HRMS-ESI (m/z) $[M + H]^+$ calcd for $C_{32}H_{27}ClNO_5^+$, 540.1572, found: 540.1571.

2.3. General procedure for the spectra measurement

General Procedure for H₂O₂ Detection

All UV-vis, fluorescence, and quantum yield measurements were carried out in 10 mM PBS buffer solution containing 5% CH₃OH, pH 7.4. In a 5 mL tube, PBS buffer (4 mL) and **ACR** (250 μ L, 100 μ M in CH₃OH) were mixed, and then H₂O₂ solution (50 μ L, 4 mM, 80 eq) was added. The final solution volume was adjusted to 5 mL with PBS buffer to obtain a final concentration of 5 μ M. After rapid mixing of the solution, it was placed for 120 min then transferred to a 10 $\times$ 10 mm quartz cell and incubated at 37 °C for *in vitro* detection. Fluorescence spectra were recorded in the range from 560 to 700 nm with λ_{ex} = 470 nm, and absolute emission quantum yields were determined accordingly.

Preparation of ROS and RNS

H₂O₂, *t*BuOOH, NaClO, NaNO₂, NaNO₃, Na₂S, Cys, Hcy, GSH, Na₂S₂O₃, KF, NaBr were prepared from the source of their respective salts or solutions in MeOH/PBS buffer, 10 mM, pH = 7.4, 5/95.

t-BuOO•

t-BuOO• was generated from 2,2'-azobis(2-amidinopropane)dihydrochloride (108.5 mg, CAS: 2997-92-4), which was dissolved in HEPES buffer (10 mL, pH 7.2) and then *t*-BuOH (36 mg) was added. Then 50 μ L of this solution was immediately added to 2 mL HEPES solution containing **ACR** (250 μ M). The resulting solution was diluted to 5 mL with HEPES buffer.

ONOO⁻

The synthesis of peroxynitrite by the addition NaNO₂ to a solution of H₂O₂ and HCl in HEPES

buffer (pH 7.2). NaNO_2 (5 mL, 0.6M) was added to a solution of H_2O_2 (0.6M) and HCl (0.7M) in HEPES buffer at 0 °C. MnO_2 (50 mg) was added to consume the surplus H_2O_2 and then filtered. The resulting solution was kept at -20 °C. The peroxynitrite concentration was determined to be 170 mM by using an extinction coefficient of $1670 \pm 50 \text{ cm}^{-1}(\text{mol/L})^{-1}$ at 302 nm. Then 12 μL of this solution was immediately added to 2 mL HEPES solution containing **ACR** (250 μM). The resulting solution was diluted to 5 mL with HEPES buffer.

OH•

Hydroxyl radicals were generated by the addition of FeSO_4 and H_2O_2 at room temperature in HEPES buffer (pH 7.2) and the mixture was then stirred for 30 min. FeSO_4 (4 mL, 100mM) and H_2O_2 (4 mL, 100mM) was added to HEPES solution, which was diluted to 10.0 mL. Then 50 μL of this solution was immediately added to 2 mL HEPES solution containing **ACR** (250 μM). The resulting solution was diluted to 5 mL with HEPES buffer.

$\text{O}_2^{\cdot-}$

Superoxide radical was prepared from the source of KO_2 at room temperature in HEPES buffer (pH 7.2). KO_2 (28 mg) was added to absolute DMSO (1 mL). The solution was diluted to 10.0 mL with HEPES solution after 10 min. Then 50 μL of this solution was immediately added to 2 mL HEPES solution containing **ACR** (250 μM). The resulting solution was diluted to 5 mL with HEPES buffer.

Quantum Yields

Fluorescence quantum yields of **ACR** were determined in PBS buffer (10 mM, pH 7.4) with rhodamine B ($\Phi = 0.89$, in ethanol) as a reference. 7'-Chloro-*N,N*-diethylrhodol was obtained in the experiment by addition of 80 eq of H_2O_2 to the solution of probe **ACR**. The quantum yields

were calculated using an Eq follows:

$$\Phi_u = [(A_s FA_u \eta^2) / (A_u FA_s \eta_0^2)] \Phi_s.$$

Where A_s and A_u are the absorbance of the reference and sample solution at the reference excitation wavelength, FA_s and FA_u are the corresponding integrated fluorescence intensity, and η and η_0 are the solvent refractive indexes of sample and reference, respectively. Absorbance of sample and reference at their respective excitation wavelengths was controlled to be lower than 0.05.

Quantum yield of **ACR**: $\Phi = 0.0122$

Quantum yield of **ACR**+H₂O₂: $\Phi = 0.8978$

Detection limit

To determine the detection limit, the emission intensity of probe **ACR** without H₂O₂ was measured by 10 times and the standard deviation of blank measurements was determined. The detection limit was then calculated with the equation: detection limit = $3\sigma/k$, where σ was the standard deviation of blank measurements, k was the slope between intensity difference versus sample concentration. According to fluorometric method, the detection limit of probe **ACR** for H₂O₂ was determined as 0.2650 μ M.

$$\text{Detection limit of ACR} = 0.06473 * 0.021578925 / 0.2443 = 0.2650$$

2.4. MTT assay

The MTT assay was used to evaluate the cytotoxicity of probe. MCF-7 cells and 3T3 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum at 37 °C in an atmosphere containing 5% CO₂. MCF-7 cells and 3T3 cells were seeded onto 96-well plates at a density of 1×10⁴ cells/well and incubated for 24 h. The medium was replaced by various probe over a range

of concentrations (0.5 μ M to 30 μ M) dissolved in culture medium. After incubation at 37 °C, 5% CO₂ for 24 h, each well of cells were treated with 20 μ l MTT solution (5 mg/mL), and incubated for another 4 h. After that, the medium was removed, and 100 μ L of DMSO were added to dissolve the formazan crystals. The plate was agitated for 10 min, and each well was finally analyzed by the microplate reader (Thermo Scientific, Multiskan FC) and detected by the absorbance at 570 nm.

MCF-7: IC₅₀ 69.6 μ M.

2.5. Cell culture and fluorescence imaging

The HeLa cells were cultured in DMEM medium supplemented with 10% fetal bovine serum at 37 °C in an atmosphere containing 5% CO₂. For live cell imaging, probe **ACR** was added to the cells and incubated for 15 min and washed with PBS (phosphate-buffered saline) three times. After replacement of the medium, cells were imaged using an Olympus (FV1000) confocal laser scanning microscope with a 200 $\times$ objective lens.

3. Results and discussion

3.1. Design and synthesis

Taken an overall view of the developed H₂O₂ probes, it was obviously that the oxidability of H₂O₂ was deeply exploited. For example, the most used H₂O₂-responsive site, boronate responded to H₂O₂ by a chemospecific oxidation of the boronate to phenol [12]. However, few of these H₂O₂ probes were based on the nucleophilicity of H₂O₂. In fact, developing probes for H₂O₂ based on a non-oxidative mechanism would allow the highly specific detection of H₂O₂ under the complicated oxidative circumstances found in biological systems. A lot of evidence showed that the nucleophilicity of H₂O₂ is just as **significant** as its oxidability [12,19,20]. Even in the oxidation of

the boronate, H_2O_2 acts as a nucleophile firstly[32]. So it is important to construct a fluorescent probe for H_2O_2 based on its nucleophilicity. In fact, it is challenging to design a H_2O_2 probe based on its nucleophilicity opposite the interference of many ROSs. However, the nucleophilicity of H_2O_2 is different from those of the ROSs after all. Therefore, it may be a feasible strategy for designing a H_2O_2 probe by linking an ester bond to a fluorophore. During screening for hydrogen sulfide fluorescent probes, a compound we named **ACR**{6'-[2-(azidomethyl)benzoyl]-7'-chloro-*N,N*-diethylrhodol} was synthesized by the reaction of 7'-chloro-*N,N*-diethylrhodol and 2-(azidomethyl)benzoyl acid (scheme 1). However, **ACR** did not respond to H_2S at all, but triggered obvious fluorescence enhancement to H_2O_2 . Considering the nucleophilicity of H_2O_2 , the detection of H_2O_2 by **ACR** was based on a non-oxidative mechanism. And **ACR** responded to H_2O_2 by a nucleophilic substitution of the ester bond.

3.2 Sensing properties of probe **ACR**

ACR exhibited almost no fluorescence (fluorescence quantum yield: $\Phi = 0.0122$, in $\text{CH}_3\text{OH}/\text{PBS}$ buffer, 10 mM, pH = 7.4, 5/95). To verify the selectivity of **ACR** for H_2O_2 , the fluorescence changes of **ACR** (5 μM) were examined upon addition of H_2O_2 (80 equiv) and other ROS (peroxynitrite, superoxide radical, nitrate radical, *t*BuOOH, hydroxy radical, NaOCl, and so on) in PBS for 0–120 min (Fig. 1A). As shown in Fig. 1A, H_2O_2 could trigger obvious fluorescent increments while other ROSs showed a negligible fluorescence response with **ACR**. *t*BuOO $\cdot$ could also lead to small fluorescence enhancement. As shown in Fig. 1B, the relative enhancement of fluorescence caused by *t*BuOO $\cdot$ was 3.4-fold, which was much lower than that of H_2O_2 (127-fold, fluorescence quantum yield: $\Phi = 0.8978$). As an important interfering species to

H₂O₂, peroxyxynitrite even lead a smaller fluorescence enhancement than *t*BuOO[•]. Superoxide radical triggered a slightly obvious fluorescence enhancement which was still far lower than that of H₂O₂. Considering **ACR** was designed for a H₂S probe firstly and 2-azidomethylbenzoate had been used as a H₂S trap [33]. Although 2-azidomethylbenzoate connected to an electron rich dye was failed to response to H₂S, it was still essential to test and verify H₂S [34]. Then H₂S and other biologically relevant nucleophilic species were also examined. As a result, probe **ACR** barely showed little fluorescence enhancement under the same condition (Fig. 1B).

Subsequently, the changes of fluorescence spectra of probe **ACR** incubated with various concentrations of H₂O₂ were monitored. Fig. 2A showed the fluorescence spectra of probe **ACR** before and after addition of different amounts of H₂O₂. As anticipated, after incubation with H₂O₂, the fluorescence intensity increased gradually. Fig. 2B shows the plot of the relative fluorescence intensity (F/F₀) at 560 nm as a function of concentration of H₂O₂. The fluorescence intensity of probe **ACR** increased almost linearly with the concentration of H₂O₂ in the range of 0-400 μM, and with a correlation coefficient of 0.9987. Good linear correlation indicated that **ACR** was able to qualitatively and quantitatively determine the level of H₂O₂.

When treated with H₂O₂, **ACR** showed a fluorescent intensity increasing gradually almost linear to the time in 120 min (Fig. 3). Indeed, the enhancement of fluorescence intensity after reaction with H₂O₂ was as high as 127-fold. By fitting the data (Figs. 3 and S1, ESI[†]) with a pseudo-first order model (**ACR**, 5 μM and H₂O₂, 400 μM), the reaction rate constant was obtained as $5.7 \times 10^{-2} \text{ s}^{-1}$ at 25 °C (Fig. S2, ESI[†]). This value is comparable to (a little smaller than) those of previously reported fluorescence probes for H₂O₂ based on boronate chemistry [35] and Baeyer-Villiger type reaction [22]. Specifically, the detection limit of H₂O₂ was determined to be

0.2650 μM based on the $3\sigma/\text{slope}$ method (Fig. S3, ESI[†]), which was much lower than those of the reported probes [12,13]. Perhaps, the special non-oxidative detection route increased the selectivity toward H_2O_2 . We then evaluated the effect of pH on the fluorescence of **ACR**, which showed it was very stable from pH 6 to 8, even in the presence of H_2O_2 (Fig. S4, ESI[†]).

Next, we performed competition experiments in the presence of ROS and H_2S (Fig. 4). **ACR** was still able to respond to H_2O_2 with strong fluorescence enhancements in the coexistence of the interfering species. Although the reaction rate constant of **ACR** with H_2O_2 was a little smaller than the previously reported probes, **ACR** exhibited excellent selectivity to H_2O_2 . Moreover, the non-oxidative process of **ACR** for detection of H_2O_2 was confirmed by the HRMS-ESI spectra. The peak for 7'-chloro-*N,N*-diethylrhodol (calcd for $[\text{M} + \text{H}]^+ [\text{C}_{24}\text{H}_{21}\text{ClNO}_4]^+$, 422.1154, found: 422.1140, Fig. S5, ESI[†]), was detected after probe **ACR** was reacted with H_2O_2 .

To further prove the selectivity of probe **ACR** for H_2O_2 , time-dependent fluorescence changes over ROS and H_2S were recorded. As shown in Fig. 5, the fluorescence enhancements for other ROS and H_2S were still negligible until 120 min. More importantly, the fluorescent intensity for H_2O_2 was increased obviously to the time, while the increment for other ROS and H_2S almost ceased. Thus, the interference of other ROS and H_2S was negligible, and probe **ACR** showed high selectivity toward H_2O_2 .

3.3 Exploring the effect of chlorine and azide

The detection mechanism of probe **ACR** was very attractive for its special non-oxidative route, which would allow the high specific detection of H_2O_2 under complicated oxidative circumstances found in biological systems [19]. Although it was believed that a nucleophilic

substitution reaction was happened during the detection, the effect of every functional group was still unclear. In fact, we have not been fully understood the nucleophilicity of H_2O_2 which hindered deep studying. A deep study was conducted for understanding the nucleophilicity of H_2O_2 . To further explore the relationship of the chlorine atom, azide group and the activity of the carbonyl, two other compounds **AR**[34] and **MCR** were synthesized and investigated. Both **AR** and **MCR** showed little response when treated with H_2O_2 or other ROS, even reactive sulfur species (RSS). The results kept the same even in 120 min (Scheme 2, Figs. S6 and S7, ESI[†]). In other words, only the ester bond in **ACR** was able to be broken by H_2O_2 , and this bond was only able broken by H_2O_2 . The results showed that probe **ACR** was made up of three essential parts, chlorine atom and azide group, which form an active carbonyl.

3.4 Detection of H_2O_2 in living cells

Encouraged by the above results, we subsequently assessed the potential applications of **ACR** for monitoring and imaging of H_2O_2 in living cells. Firstly, the cytotoxicity of **ACR** was evaluated using A-549 cells and Raw 264.7 cells by MTT assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Fig. S8, ESI[†]]. Probe **ACR** showed almost no cytotoxicity in the 0.1–30 μM range to both of them, implying that the probe was suitable for bioimaging of H_2O_2 in living cells. HeLa cells incubated with **ACR** (10 μM) in culture medium for 15 min at 37 °C, showed almost no fluorescence (Fig. 6B). Fig. 6A is the bright-field image. Fig. 6C is the overlay of the Figs. 6A and B. However, if HeLa cells were pretreated with **ACR** (10 μM) for 15 min and then incubated with H_2O_2 (25 μM) for 15 min, strong fluorescence was observed (Fig. 6D). This result indicates that probe **ACR** has the potential

to visualize H_2O_2 levels in living cells. Fig. 6E is the overlay of the Figs. 6D and F (bright-field image). The obvious fluorescent increments indicated that the probe could image of the exogenous H_2O_2 .

4. Conclusion

In summary, aiming at finding high-selectivity fluorescent probes for detection of H_2O_2 , a new probe **ACR** was designed and synthesized. Probe **ACR** was proved to be made up of three essential parts, chlorine atom, azide group and an active carbonyl, due to the special nucleophilicity of H_2O_2 . This probe showed high selectivity for H_2O_2 and a linear fluorescence intensity enhancement with a wide range of concentrations of H_2O_2 . Preliminary fluorescence imaging experiments in HeLa cells indicated its potential to image H_2O_2 in biological systems (endogenous levels of H_2O_2 are reported to be less than 100 μM) [25]. Relevant studies on this strategy and its biological applications are underway.

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List of Figure Captions

Scheme 1. Synthesis of probe **ACR** and the response of **ACR** to H_2O_2 .

Scheme 2. Confirmation the effects of chlorine atom and azide group in probe **ACR**.

Fig.1.(A) Fluorescence response of **ACR** (5 μM) incubated with ROS and H_2O_2 (80 eq) in $\text{CH}_3\text{OH}/\text{PBS}$ buffer (10 mM, pH = 7.4, 5/95) for 120 min. (B) Bar graph. 1. Blank, 2. H_2O_2 , 3. $t\text{BuOOH}$, 4. $t\text{BuOO}\cdot$ (in HEPES buffer), 5. $\text{HO}\cdot$ (in HEPES buffer), 6. NaClO , 7. ONOO^- (in HEPES buffer), 8. NaNO_2 , 9. $\text{O}_2^{\cdot-}$ (in HEPES buffer), 10. NaNO_3 , 11. Na_2S , 12. Cys, 13. Hcy, 14. GSH, 15. $\text{Na}_2\text{S}_2\text{O}_3$, 16. KF, 17. NaBr, 18. KI. $\lambda_{\text{ex}} = 470 \text{ nm}$, $\lambda_{\text{em}} = 560 \text{ nm}$. Slits: 5/5 nm.

Fig.2.(A) Fluorescence spectra of **ACR** (5 μM) incubated with different concentrations of H_2O_2 (0-200 eq, 0-1.0 mM) in $\text{CH}_3\text{OH}/\text{PBS}$ buffer (10 mM, pH = 7.4, 5/95) for 120 min. (B) Linear fitting chart. $\lambda_{\text{ex}} = 470 \text{ nm}$, $\lambda_{\text{em}} = 560 \text{ nm}$. Slits: 5/5 nm.

Fig.3. (A) Time-dependent fluorescence spectral changes of **ACR** (5 μM) with H_2O_2 (80 eq, 400 μM) in $\text{CH}_3\text{OH}/\text{PBS}$ buffer (10 mM, pH = 7.4, 5/95). (B) Line chart. $\lambda_{\text{ex}} = 470 \text{ nm}$, $\lambda_{\text{em}} = 560 \text{ nm}$. Slits: 5/5 nm.

Fig.4. Fluorescence responses of **ACR** (5 μM) towards H_2O_2 (400 μM) with various analytes (400 μM) in $\text{CH}_3\text{OH}/\text{PBS}$ buffer (10 mM, pH = 7.4, 5/95). 1. H_2O_2 only; 2-10, H_2O_2 and indicated analyte: 2. $t\text{BuOOH}$, 3. $t\text{BuOO}\cdot$ (in HEPES buffer), 4. $\text{HO}\cdot$ (in HEPES buffer), 5. ONOO^- (in HEPES buffer), 6. NaNO_2 , 7. $\text{O}_2^{\cdot-}$ (in HEPES buffer), 8. Na_2S , 9. Cys, 10. Hcy, 11. KF. $\lambda_{\text{ex}} = 470 \text{ nm}$, $\lambda_{\text{em}} = 560 \text{ nm}$. Slits: 5/5 nm.

Fig.5. Time-dependent fluorescence spectral changes of **ACR** (5 μM) with ROS and H_2S (80 eq for each, 400 μM) in $\text{CH}_3\text{OH}/\text{PBS}$ buffer (10 mM, pH = 7.4, 5/95). Time points represent 30, 60, 90, and 120 min. $\lambda_{\text{ex}} = 470 \text{ nm}$, $\lambda_{\text{em}} = 560 \text{ nm}$. Slits: 5/5 nm.

Fig.6. Bright-field (A), fluorescence image (B) and the overlay (C) of Figs. A and B of HeLa cells incubated with **ACR** (10 μ M) for 15 min. Bright-field (D), fluorescence image (E) and the overlay (F) of Figs. D and E of HeLa cells incubated with **ACR** (10 μ M) for 15 min and washed with PBS three times. After replacement of the medium, cells were incubated with H₂O₂ (25 μ M) for another 15 min.