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Invited feature article

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 H_2O_2 was developed. An ester bond was broken by the nucleophilic substitution of H_2O_2 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 H_2O_2 and a linear fluorescence intensity enhancement with a wide range of concentrations of H_2O_2. Fluorescence imaging experiments in HeLa cells indicated its potential to image H_2O_2 in biological systems.

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 $Si(CH_3)_4$ as an internal standard. NMR spectra were recorded in $CDCl_3$ or DMSO-$_{d6}$. 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.

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2.2. Synthesis

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

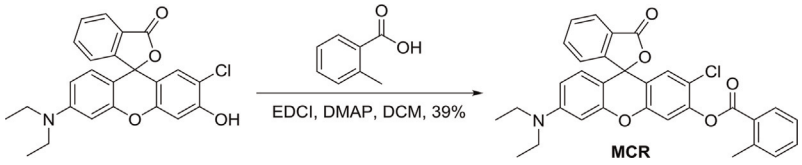
To a suspension of 2-(4-diethylamino-2-hydroxybenzoyl)benzoyl acid (6.0 g, 19.1 mmol) and resorcinol (2.1 g, 19.1 mmol) in trifluoroacetic acid (TFA, 20 mL) in a sealing tube, was added molecular sieve (2.0 g). The resulting mixture was heated to 90 °C and stirred for 6 h. Then the mixture was cooled 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); ^{1}H NMR (400 MHz, DMSO-$_{d6}$) 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) [M+H]$^+$ calcd for $C_{24}H_{21}ClNO_4^+$, 422.1154, found: 422.1151; mp 177.9 ~ 179.5 °C.

Synthesis of **ACR**

A solution of 2-(azidomethyl)benzoyl acid (252.2 mg, 1.4 mmol) in DCM (5 mL), was added 1-*E*thyl-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); ^{1}H NMR (400 MHz, $CDCl_3$) ^{1}H 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) νmax 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-methylbenzoyl 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); ^{1}H 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

2.3.1. General procedure for H_2O_2 detection

All UV–vis, fluorescence, and quantum yield measurements were carried out in 10 mM PBS buffer solution containing 5% CH_3OH, pH 7.4. In a 5 mL tube, PBS buffer (4 mL) and **ACR** (250 μL, 100 μM in CH_3OH) were mixed, and then H_2O_2 solution (50 μL, 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 μM. After rapid mixing of the solution, it was placed for 120 min then transferred to a 10 × 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.

2.3.2. Preparation of ROS and RNS

H_2O_2,*t*BuOOH, NaClO, $NaNO_2$, $NaNO_3$, Na_2S, Cys, Hcy, GSH, $Na_2S_2O_3$, KF, NaBr were prepared from the source of their respective salts or solutions in MeOH/PBS buffer, 10 mM, pH = 7.4, 5/95.

2.3.3. 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, pH7.2) and then *t*-BuOH (36 mg) was added. 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.