Chapter 5

Synthetic Fluorescent Probes for Imaging of Peroxynitrite and Hypochlorous Acid in Living Cells

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Abstract

Peroxynitrite ($ONOO^-$) and hypochlorous acid (HOCl) are two highly reactive oxygen species generated in biological systems. The overproduction of peroxynitrite or hypochlorous acid is implicated in a broad array of human pathologies including vascular, immunological, and neurodegenerative diseases. However, unambiguous detection of these reactive oxygen species has been relatively difficult due to their short biological half-lives and multiple reaction pathways. Based on their specific chemical reactions, we have developed fluorescent probes **HKGreen-1** and **HKOCl-1** for highly sensitive detection of peroxynitrite and hypochlorous acid, respectively. Both probes have been demonstrated to be able to discriminate corresponding reactive species from other reactive oxygen and nitrogen species (ROS and RNS) in not only chemical systems but also biological systems. The endogenous production of peroxynitrite in neuronal cells under oxygen-glucose deprivation (OGD) conditions has been visualized for the first time by utilizing **HKGreen-1** probe, whilst the endogenous production of hypochlorous acid in macrophage cells upon stimulation with LPS, IFN-γ, and PMA has been imaged by utilizing **HKOCl-1** probe.

Key words: Fluorescent probe, **HKGreen-1**, **HKOCl-1**, peroxynitrite, hypochlorous acid, reactive oxygen species, reactive nitrogen species, neuronal cell, oxygen-glucose deprivation, macrophage cell.

1. Introduction

Peroxynitrite ($ONOO^-$), a short-lived highly reactive species, is formed in vivo by the diffusion-controlled reaction between overproduced nitric oxide ($^{\bullet}NO$) and superoxide ($O_2^{\bullet -}$) radicals (1–4). Accumulating evidence has suggested that peroxynitrite

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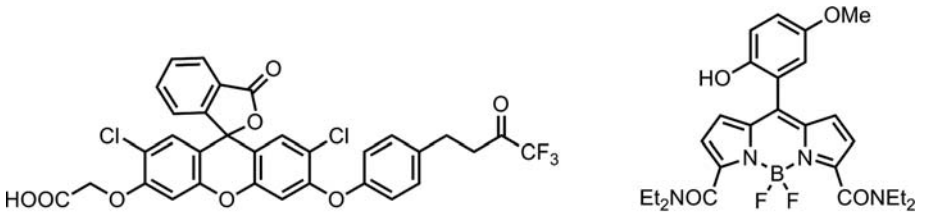
contributes to the pathogenesis of many human diseases including ischemic reperfusion injury, circulatory shock, diabetes, chronic heart failure, stroke, myocardial infarction, cancer, chronic inflammatory diseases, and neurodegenerative disorders (5, 6). Unfortunately, comprehensive investigations and understandings of the pathological and physiological roles of peroxynitrite have been hampered by the difficulty in achieving specific detection of intracellular peroxynitrite for its short biological half-life and multiple reaction pathways (7). Despite numerous efforts in the literature to develop new methods for the detection of peroxynitrite, none of them turns out to be specific and highly sensitive (7–9).

On the basis of a specific chemical reaction (10–12), we have developed a novel fluorescent probe **HKGreen-1** (**Fig. 5.1**) for highly sensitive detection of peroxynitrite (12). The probe **HKGreen-1** has been proven to be applicable for the detection and imaging of peroxynitrite in living cells (12, 13). Probe-loaded primary cultured neuronal cells displayed strong fluorescence signals after treatment with peroxynitrite donor 3-morpholinosydnonimine hydrochloride (SIN-1), whereas negligible fluorescence signals were observed in the cells upon treatment with nitric oxide donor *S*-nitroso-*N*-acetyl-*DL*-penicillamine (SNAP) or superoxide donor (xanthine/xanthine oxidase). Furthermore, by utilizing **HKGreen-1** probe, visualization of endogenous peroxynitrite production in neuronal cells was achieved for the first time under oxygen-glucose deprivation (OGD) conditions (14, 15).



HKGreen-1 **HKOCl-1**

Fig. 5.1. Chemical structures of fluorescent probes **HKGreen-1** (12) and **HKOCl-1** (24).

Hypochlorous acid (HOCl, pK_a = 7.5) or its basic form, hypochlorite (OCl^-), is another reactive species present in cells. It is generated predominantly in activated leukocytes, including neutrophils, macrophages, and monocytes (16), by myeloperoxidase (MPO)-catalyzed oxidation of chloride ions in the presence of hydrogen peroxide. In biological systems, hypochlorous acid functions mainly in the prevention of microbial invasion as neither bacteria nor mammalian cells can neutralize its toxic effects due to a lack of enzymes for catalytic detoxification (17). Many studies have pointed to the role of hypochlorous acid in the pathogenesis

of human diseases including cardiovasculopathies, osteoarthritis, and neurodegeneration (16, 18–21). Two red fluorescent probes for hypochlorous acid were reported recently (22, 23), though issues concerning their sensitivity remain to be addressed. We have developed a green fluorescent probe **HKOCl-1** (**Fig. 5.1**) for highly sensitive and selective detection of hypochlorous acid based on its specific reaction with *p*-methoxyphenol (24). By utilizing **HKOCl-1** probe, endogenous production of hypochlorous acid in macrophage cells was visualized after exposure to stimuli such as lipopolysaccharide (LPS), interferon-γ (IFN-γ), and phorbol 12-myristate 13-acetate (PMA) (24).

2. Materials

2.1. Cell Culture

1. Pregnant Sprague-Dawley rats (Harlan).
2. Neuronal cell growth medium: Neurobasal medium (Gibco) supplemented with 2% B27 (Gibco), 0.5 mM glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin (all Sigma-Aldrich).
3. Neurobasal medium (Gibco) supplemented with 2% B27 minus antioxidants (B27 minus AO; Gibco).
4. Oxygen-glucose deprivation (OGD) medium: Dulbecco's modified Eagle's medium (DMEM) without glucose (Gibco) gassed with 94% N_2/1% O_2/5% CO_2 for 15 min.
5. Macrophage cell growth medium: Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 10% fetal bovine serum (FBS; Gibco) supplemented with 100 units/mL penicillin (Sigma-Aldrich) and 100 μg/mL streptomycin (Sigma-Aldrich).
6. RAW 264.7 macrophage cells (ATCC).
7. T-75 culture flasks (Corning).
8. Teflon cell scrapers (Thermo Fisher).
9. Poly-L-lysine-coated 6-well culture plates (BD Biosciences).
10. 35 mm cover-slip dishes (MatTek).
11. Phosphate-buffered saline (PBS; 1×, Gibco).

2.2. Imaging of Peroxynitrite Production with Probe HKGreen-1

1. Acetonitrile (CH_3CN; ≥99.93%, biotech. grade, Sigma-Aldrich).
2. Dimethyl sulfoxide (DMSO; ≥99.9%, for molecular biology, Sigma-Aldrich).

3. **HKGreen-1** is synthesized according to published procedures (12). Prepare a 2 mM stock solution of **HKGreen-1** (F.W. = 659.39) in CH_3CN by dissolving 2.0 mg of the probe crystals in 1.517 mL of anhydrous acetonitrile (*see* **Note 1**).
4. Dihydroethidium (HE; Invitrogen). Prepare a 4 mM stock solution of HE (F.W. = 315.41) in DMSO by dissolving 1.3 mg of HE in 1.030 mL of anhydrous DMSO (*see* **Note 2**).
5. 3-Morpholinosydnonimine hydrochloride (SIN-1; Sigma-Aldrich), dissolved at 1 mM in water immediately before the experiment.
6. *S*-Nitroso-*N*-acetyl-*DL*-penicillamine (SNAP; Sigma-Aldrich), dissolved at 1 mM in DMSO immediately before the experiment.
7. Xanthine and xanthine oxidase (X/XO; Sigma-Aldrich).

2.3. Imaging of Hypochlorous Acid Production with Probe HKOCl-1

1. *N,N*-dimethylformamide (DMF; ≥99.93%, biotech. grade, Sigma-Aldrich).
2. **HKOCl-1** is synthesized according to published procedures (24). Prepare a 2 mM stock solution of **HKOCl-1** (F.W. = 512.24) in DMF by dissolving 1.0 mg of the probe crystals in 0.976 mL of anhydrous DMF (*see* **Note 3**).
3. Lipopolysaccharide (LPS; purified by phenol extraction, Sigma-Aldrich), dissolved at 1 mg/mL in 1× PBS immediately before the experiment.
4. Interferon-γ (IFN-γ; ≥98%, Sigma-Aldrich), dissolved at 50 μg/mL in 1× PBS immediately before the experiment.
5. Phorbol 12-myristate 13-acetate (PMA; ≥99%, molecular biology grade, Sigma-Aldrich), dissolved at 20 μg/mL in DMSO immediately before the experiment.

3. Methods

3.1. Imaging of Exogenous Peroxynitrite with HKGreen-1 in Primary Cultured Neuronal Cells

1. Sprague-Dawley rats were used with permission from local health authorities and maintained in compliance with the principles set forth in the "Guide for Care and Use of Laboratory Animals." Primary cortex neurons from embryonic day 15 Sprague-Dawley rats were isolated as described in the literature (14, 25).
2. Seed the suspension of dispersed neuronal cells at a density of 2×10^6 cells/well on poly-L-lysine coated 6-well

culture plates or 35 mm cover-slip dishes with neuronal cell growth medium. Maintain the cells in a humidified incubator at 37°C, in 5% CO_2-95% air. After 4 days, replace half of the growth medium with fresh medium. Change growth medium twice a week. On day 9, change growth medium to neurobasal medium supplemented with B27 minus AO. On day 10, use cultured cortical cells for imaging experiments.

3. Retrieve plates or dishes from the incubator. Discard the old medium. Add fresh medium (1.980 mL; neurobasal medium supplemented with 2% B27 minus AO) and incubate the plates or dishes for at least 1 h.
4. Add 20 μL of **HKGreen-1** stock solution to each well to give a final concentration of 20 μM in the medium. Incubate for 15 min at 37°C to load cells with the probe (*see* **Note 4**).
5. Take out the cells from the incubator and wash them with 1× PBS three times. Treat the cells with 10 μM SIN-1 or 10 μM SNAP or 100 μM of xanthine plus 0.1 IU of xanthine oxidase (X/XO) in 2 mL PBS for 15 min (*see* **Notes 5** and **6**).
6. Wash the cells again with 1× PBS. After washing, add 2 mL of 1× PBS to each sample (*see* **Note 5**).
7. Observe cells under a fluorescent microscope. Use bright-field illumination to obtain phase-contrast images of cells after focusing on them. Record fluorescence images by using excitation at 488 nm. Examples of the fluorescence images are shown in **Fig. 5.2** (*see* **Notes 7** and **8**).

3.2. Imaging of Endogenous Peroxynitrite Production with HKGreen-1 in Primary Cultured Neuronal Cells Under Hypoxia Conditions

1. Prepare neuronal cells on imaging plates or dishes (*see* **Section 3.1**). Change to fresh medium (1.978 mL) 1 h before the experiments.
2. Add 20 μL of **HKGreen-1** and 2 μL of HE stock solutions to each well to give final concentrations of 20 and 4 μM, respectively. Incubate for 15 min at 37°C to load cells with the probes.
3. Take out the cells from the incubator and gently wash them with 1× PBS twice. Then incubate the cells in OGD medium (2 mL) at 37°C for indicated time (2 h or 12 h) in a humidified hypoxia chamber equilibrated with 94% N_2/1% O_2/5% CO_2.
4. At the end of OGD, wash the cells twice with 1× PBS and observe them under a fluorescent microscope. Use bright-field illumination to obtain phase-contrast images of cells after focusing on them. Record fluorescence images by using excitation at 488 nm. Detect green fluorescence at 510 nm produced by **HKGreen-1** oxidation and the red

Fig. 5.2. Imaging of primary cultured neuronal cells: phase contrast (*left*) and fluorescence (*right*). Neuronal cells were incubated with **HKGreen-1** (20 μM) for 15 min and then subjected to different treatments: (**a**) Control; (**b**) 10 μM of SIN-1; (**c**) 10 μM of SNAP; (**d**) 100 μM of xanthine plus 0.1 IU of xanthine oxidase. (Reprinted with permission from (12), Copyright 2006 American Chemical Society.).

fluorescence at 590 nm produced by HE oxidation. Examples of fluorescence images are shown in **Fig. 5.3** (*see* **Notes 7**, **8**, and **9**).

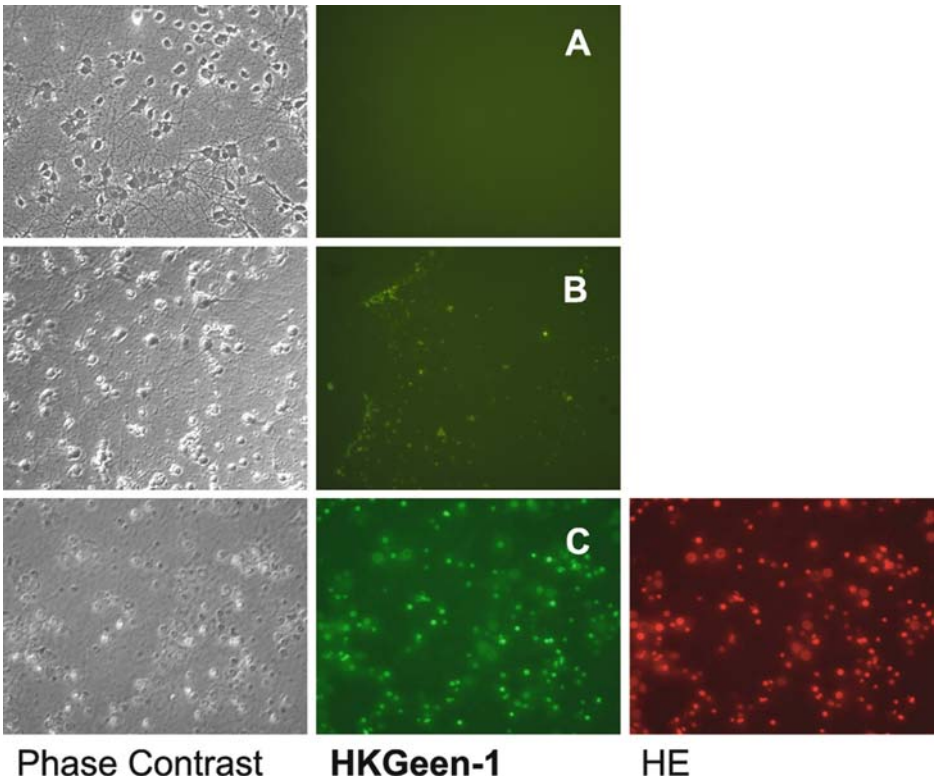


Fig. 5.3. Imaging of primary cultured neuronal cells: phase contrast (*left*); fluorescence via green channel (*middle*); fluorescence via red channel (*right*). Neuronal cells were incubated with **HKGreen-1** and HE (20 μM and 4 μM, respectively) for 15 min and then subjected to OGD condition: (**a**) Control; (**b**) OGD for 2 h; (**c**) OGD for 12 h.

3.3. Imaging of Endogenous Hypochlorous Acid Production with HKOCl-1 in Macrophage Cells

1. Culture RAW 264.7 macrophage cells in DMEM containing 10% FBS supplemented with 100 U/mL of penicillin and 100 μg/mL streptomycin at 37°C in 5% CO_2. Subculture cells by scraping and seeding them in T-75 flasks. Change growth medium every two or three days. Grow cells to confluence for imaging experiments.
2. Seed macrophage cells at a density of 2×10^6 cells/well on 35 mm cover-slip dishes with serum-free DMEM. After 24 h, change to fresh serum-free DMEM (1.996 mL) and incubate the cells for 1 h.
3. Take out the cells from the incubator. Add 2 μL of LPS stock solution and 2 μL of IFN-γ stock solution (final concentrations of 1 μg/mL and 50 ng/mL in medium, respectively) and incubate cells for further 4 h (*see* **Note 10**).

4. Take out the cells from the incubator. Add 1 μL of PMA stock solution (final concentration 20 μg/mL) and incubate for 30 min (*see* **Note 11**).
5. Add 20 μL of **HKOCl-1** stock solution to each well to give a final concentration of 20 μM in the medium and incubate for 1 h to load cells with the probe (*see* **Note 12**).
6. Take out the cells from the incubator. Wash them gently with 1× PBS twice and then add 1 mL of 1× PBS.
7. Observe the cells under a fluorescent microscope. Use bright-field illumination to obtain phase-contrast images of cells after focusing on them. Record green fluorescence images by using excitation at 488 nm. Examples of fluorescence images are shown in **Fig. 5.4** (*see* **Note 7**).

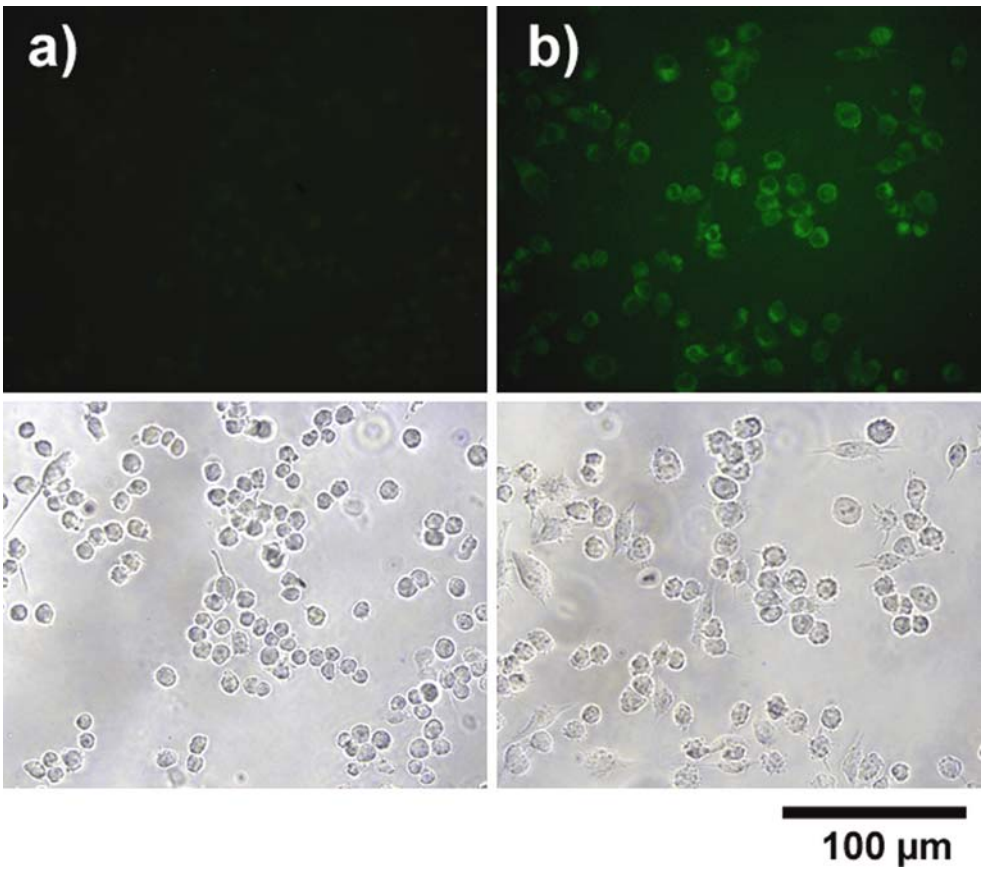


Fig. 5.4. Images of RAW 264.7 macrophages: phase contrast (*lower*); fluorescence (*upper*). Cells were treated with various stimulants and then incubated with **HKOCl-1** (20 μM) for 1 h. (**a**) Control; (**b**) LPS (1 μg/mL) and IFN-γ (50 ng/mL) for 4 h, then PMA (10 nM) for 0.5 h. (Reprinted with permission from (24), Copyright 2008 American Chemical Society.)

4. Notes

1. Crystalline form of probe **HKGreen-1** can be kept in a glass vial wrapped with aluminum foil for several months at –20°C. Use CH_3CN of high purity to make stock solutions, which can be stored at –20°C in the dark for no more than 3 weeks. Note that CH_3CN is harmful and highly flammable.
2. Use DMSO of high purity to make HE stock solutions, which can be stored at –20°C in the dark for no more than 3 weeks (26).
3. Crystalline form of probe **HKOCl-1** can be kept in a glass vial wrapped with aluminum foil for several months at –20°C. Use DMF of high purity to make stock solutions, which can be stored at –20°C in the dark for no more than 3 weeks. Note that DMF is harmful.
4. Incubation time and concentration of **HKGreen-1** need to be carefully optimized, since optimal conditions may vary for different cell types.
5. Warm PBS solution to 37°C before use and wash the cells gently.
6. Use freshly prepared solutions of SIN-1, SNAP, xanthine, and xanthine oxidase. SIN-1 solid should be kept at –20°C. Purchase SIN-1 in small packages as its quality declines after opening. The duration of incubation of cells with SIN-1 need to be optimized since simultaneous generation of superoxide and nitric oxide from SIN-1 is slow.
7. Image cells immediately after washing them with PBS. Choose healthy cells based on their morphology for imaging.
8. Adjust illumination intensity and exposure time for the sample to minimize photobleaching of **HKGreen-1**.
9. Duration of light exposure and image acquisition of HE probe should be minimized to prevent HE photooxidation, which can generate fluorescent products (26).
10. Stock solutions of LPS can be stored at 4°C in the dark for no more than 2 months. Stock solutions of IFN-γ can be stored at –20°C in the dark for no more than 2 months.
11. Stock solutions of PMA can be stored at –20°C in the dark for no more than 2 months.
12. Incubation time and concentration of **HKOCl-1** need to be carefully optimized, since optimal conditions may vary for different cell types.

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