ChemComm

RSC Publishing

COMMUNICATION

View Article Online
View Journal | View Issue

Rhodamine cyclic hydrazide as a fluorescent probe for the detection of hydroxyl radicals†

Cite this: *Chem. Commun.*, 2013, **49**, 7959

Received 20th June 2013,
Accepted 11th July 2013

DOI: 10.1039/c3cc44627a

www.rsc.org/chemcomm

Minjeong Kim, Sung-Kyun Ko, Hyemi Kim, Injae Shin* and Jinsung Tae*

A new rhodamine fluorescent probe for monitoring •OH has been developed based on the oxidative C–H abstraction reaction of rhodamine cyclic hydrazide. The probe exhibits excellent selectivity for •OH with virtually no interference by other ROS/RNS species. Fluorescent imaging of A549 and RAW264.7 cells is also successfully demonstrated to detect intracellular •OH in live cells.

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are associated with numerous pathological processes including neurodegenerative disease, ischemic or traumatic brain injury, cancer, diabetes, liver injury, and AIDS.[1,2] Therefore, over the past decade, a lot of fluorescent and luminescent detection methods have been developed for the detection of ROS/RNS, such as hydroxyl radicals (•OH), hydrogen peroxide (H_2O_2), superoxide radicals ($^{\bullet}O_2^-$), hypochlorous acid (HOCl), singlet oxygen ($^1O_2$), nitric oxide (NO•), and peroxynitrite ($ONOO^-$).[1] The hydroxyl radical (•OH), one of the most deleterious intracellular ROS, can cause severe damage to essentially all biomolecules due to its high reactivity.[3] Thus, the ability to detect and quantify intra- and extracellular •OH could provide key information about the location and the extent of redox damage and cellular responses.

Recently, several fluorescent probes for the detection of •OH have been reported.[4] Among them, the fluorescent probes based on the characteristic reactions of •OH, such as aromatic addition, spin trap, and hydrogen abstraction, show relatively good selectivity over other ROS. For example, the weakly fluorescent coumarin-3-carboxylic acid probes react with •OH to produce the fluorescent 7-HO-coumarin-3-carboxylic acid derivatives by aromatic addition reaction.[5] The spin trapping method of nitroxyl radical probes with •OH in DMSO has also been developed, where the methyl radical (•Me) generated by the reaction of DMSO and •OH traps the nitroxyl radicals which are conjugated with various fluorophores.[6] And the oxidation reaction of the hydrocyanine probes to the corresponding cyanine dyes by oxidative C–H hydrogen abstraction reaction has been applied to detect •OH and $^{\bullet}O_2^-$.[7] Alternatively, O–H hydrogen abstraction reaction by •OH and ONO_2^- could trigger deprotection of 2-[6-(4′-hydroxy)phenoxy-3*H*-xanthen-3-on-9-yl]benzoic acid to furnish the strongly fluorescent fluorescein molecule.[4a] Although these known fluorescent probes for •OH show relatively good selectivity, elimination of the interferences by other ROS is crucial for precise cellular imaging.

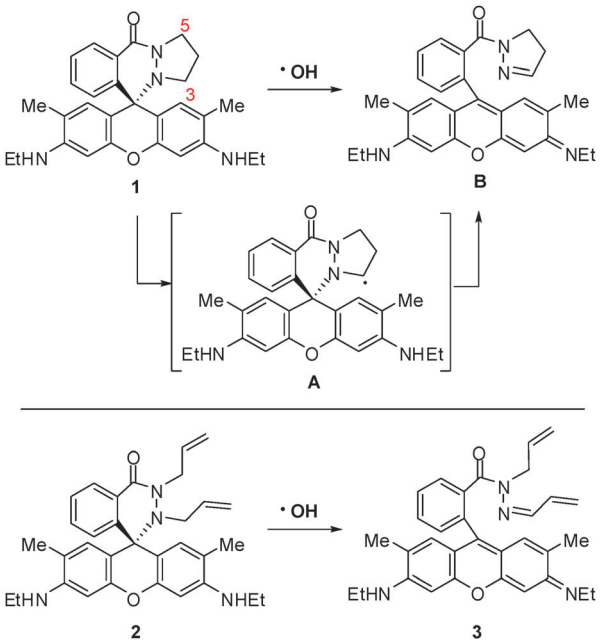
Herein, we report a highly sensitive and selective fluorescent rhodamine cyclic hydrazide probe for •OH by employing C–H hydrogen abstraction reaction of hydrazine derivatives. The •OH-mediated C–H abstraction reaction of the pyrazolidine moiety of probe **1** is expected to proceed at the most electron rich 3-position to generate the radical intermediate **A**. The spirocycle in **A** will eventually open to form the strongly fluorescent compound **B** as shown in Scheme 1.

Probe **1**[8] was prepared from Rhodamine 6G in three steps according to the literature procedure (**1**, NaOH, H_2O–EtOH; **2**, $POCl_3$, CH_2Cl_2; **3**, pyrazolidine, Et_3N, CH_2Cl_2).[9] Probe **1** forms a colorless solution in Tris–HCl buffer at pH 7.4 and shows negligible fluorescence, indicating that it exists in the spirocyclic form predominantly as expected. Upon treatment with 1.0 equiv. of •OH, probe **1** (10 μM) in Tris–HCl buffer (DMF 1% v/v) solution shows strong fluorescence at 550 nm and pink-red color in less than 2 minutes. This observation indicates that the •OH-induced reaction of probe **1** takes place rapidly at room temperature and opens the spirocyclic ring system. We attempted to detect the ring-opened product **B**, however, we were unable to isolate compound **B**. Thus, we then examined the same ring-opening reaction with probe **2** which would yield the stable conjugated imine product **3** (Scheme 1). Fortunately, we could fully characterize **3** to confirm the proposed reaction mechanism, but the initial product **3** seemed to decompose under the reaction conditions to give low isolated yield (see ESI†).

Fluorescence intensity changes of **1** upon additions of •OH (1.0 equiv.) and other ROS/RNS (5.0 equiv. of H_2O_2, ROO•, NO•,

Department of Chemistry, Yonsei University, Seoul 120-749, Korea. E-mail: jstae@yonsei.ac.kr, injae@yonsei.ac.kr; Fax: +82 2-364-7050; Tel: +82 2-2123-2603

† Electronic supplementary information (ESI) available: Experimental procedures for the synthesis and copies of ^{1}H NMR and ^{13}C NMR of **1–3**, data for UV-vis, fluorescence titration of **1**, and other data. See DOI: 10.1039/c3cc44627a



Scheme 1 Hydrogen abstraction of rhodamine probes by •OH.

$^{\bullet}O_2^-$, HOCl, $ONOO^-$, $^1O_2$) in Tris–HCl buffer (DMF 1% v/v) at pH 7.4 are shown in Fig. 1a and b. Only •OH leads to a dramatic enhancement in fluorescence intensity, but other ROS/RNS species develop no significant fluorescence intensity changes. In addition to the fluorescent changes, the colorless to pink-red color changes associated with the reaction of probe **1** with •OH are readily detectable visually (Fig. 1c).

Then, we examined the ability of **1** to quantify •OH *in vitro*. When probe **1** was treated with •OH generated by Fenton reaction,[10] the fluorescence intensity was linearly proportional to the molar equivalents of •OH (Fig. 2a). In other way, increasing H_2O_2 concentrations in Fenton's reagent with fixed amounts of $FeSO_4$ (10 μM) also linearly enhanced the fluorescence intensity of **1** (Fig. 2b). Probe **1** showed strong fluorescence

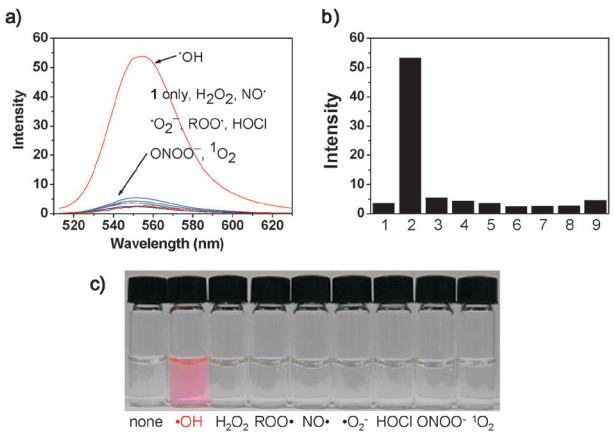


Fig. 1 (a) Fluorescence intensity changes of **1** (10 μM) upon addition of •OH (1.0 equiv.) and other ROS/RNS (5.0 equiv.) in Tris–HCl buffer (DMF 1% v/v) at pH 7.4 (25 °C, excitation at 500 nm). (b) Fluorescence intensity changes at 550 nm: 1, none; 2, •OH; 3, H_2O_2; 4, ROO•; 5, NO•; 6, $^{\bullet}O_2^-$; 7, HOCl; 8, $ONOO^-$; 9, $^1O_2$. (c) Color changes of **1** (20 μM) in the presence of ROS/RNS (5.0 equiv.).

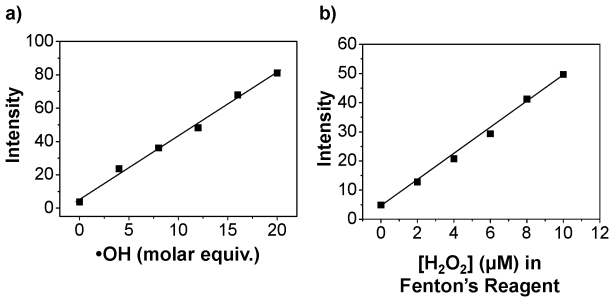


Fig. 2 Sensitivity of **1** towards •OH. Fluorescence titration of **1** (10 μM) in Tris–HCl (DMF 1% v/v) at 550 nm. (a) With increasing molar equiv. of •OH. (b) With increasing $[H_2O_2]$ in Fenton's reagent with fixed $FeSO_4$ (10 μM).

in the presence of •OH at pH 4–10 (see ESI†), therefore, we then investigated biological imaging of intracellular hydroxyl radicals.

Thus, we performed cell experiments to evaluate its potential utility for fluorescent bioimaging of hydroxyl radicals. In this study, A549 cells (human lung adenocarcinoma epithelial cells) and RAW264.7 cells (murine macrophage-like cells) were incubated with 20 μM probe **1** for 1 h. After being washed with PBS to remove the remaining probe **1**, the cells were further treated with 20 μM of Fenton's reagent ($FeSO_4 : H_2O_2$ = 1 : 10) to generate hydroxyl radicals.[11,4g] Confocal microscopy analysis reveals that the cells treated with Fenton's reagent exhibit strong red fluorescence but the cells incubated with probe **1** alone display very weak fluorescence (top panels in Fig. 3a and b). When cells were exposed to the 20 μM probe and a membrane-permeable radical scavenger (5 mM TEMPOL, 4-hydroxy-2,2,6,6-tetramethyl piperidine-*N*-oxyl)[12] prior to incubation with 20 μM of Fenton's reagent, remarkably attenuated fluorescence was observed in the treated cells (bottom panels in Fig. 3a and b), revealing that

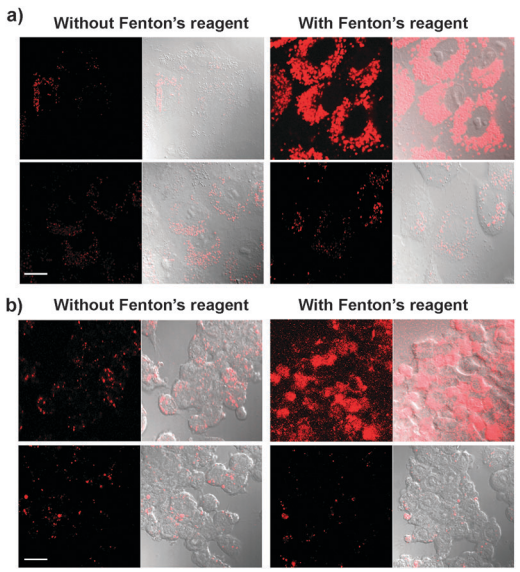


Fig. 3 Fluorescence images of cells incubated with probe **1** and Fenton's reagent. (a) A549 and (b) RAW264.7 cells were incubated with 20 μM probe **1** for 1 h, and then incubated for 1 h with 20 μM of Fenton's reagent in the absence (top panels) or the presence (bottom panels) of 5 mM TEMPOL (left; fluorescence microscope images, right; merged bright field images). Scale bar = 10 μm.

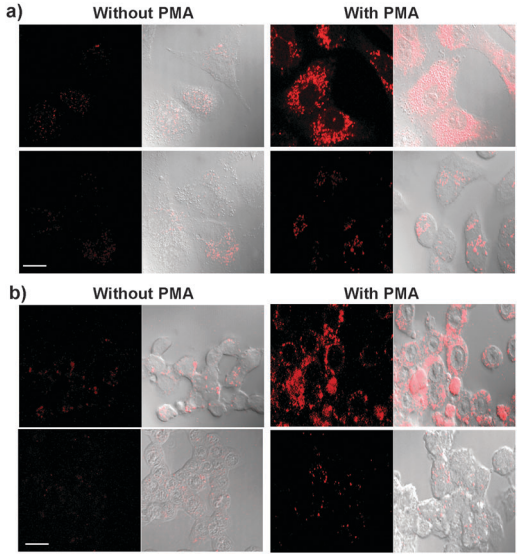


Fig. 4 Fluorescence images of cells incubated with probe **1** and PMA. (a) A549 and (b) RAW264.7 cells were incubated with 20 μM probe **1** for 1 h, and then incubated for 1 h with 10 ng mL^{-1} of PMA in the absence (top panels) or the presence (bottom panels) of 5 mM TEMPOL (left; fluorescence microscope images, right; merged bright field images). Scale bar = 10 μm.

the fluorescence signal is a consequence of the response to intracellular hydroxyl radicals.

Encouraged by the fluorescent imaging of hydroxyl radicals generated by Fenton's reagent in live cells, we then examined whether probe **1** can sense hydroxyl radicals which are produced by the physiological stimulation with phorbol 12-myristate 13-acetate (PMA).[4f,6] In this study, A549 and RAW264.7 cells were treated with 20 μM probe **1** for 1 h. After being washed with PBS to remove the remaining probe **1**, the cells were incubated with 10 ng mL^{-1} PMA to generate hydroxyl radicals. In contrast to cells untreated with PMA, a marked increase in fluorescence was observed in both cells after stimulation with PMA (top panels in Fig. 4a and b). To confirm that fluorescence turn-on was due to the response of probe **1** to the generated hydroxyl radicals, cells treated with probe **1** were incubated with 5 mM TEMPOL prior to stimulation with PMA. It was found that a radical scavenger remarkably reduced the red fluorescence in both cells (bottom panels in Fig. 4a and b), indicating that the probe monitors hydroxyl radicals generated by the physiological stimulation. These cell experiments demonstrate that the probe is cell membrane-permeable and is useful to effectively detect intracellular hydroxyl radicals in live cells.

In conclusion, we have developed a new Rhodamine 6G fluorescent probe **1** for monitoring $^{\bullet}$OH. The mechanism for sensing $^{\bullet}$OH is based on the oxidative C–H abstraction reaction of rhodamine cyclic hydrazide, which is the first example in the rhodamine fluorescent probes. Probe **1** exhibits excellent selectivity for $^{\bullet}$OH with virtually no interference by other ROS/RNS species. Fluorescent imaging of A549 and RAW264.7 cells is also successfully demonstrated to detect intracellular $^{\bullet}$OH in live cells. We expect that this imaging technique will serve as a practical tool for $^{\bullet}$OH-related biological studies.

This work was supported by Basic Science Research (No. 2011-0007015) and National Creative Research Initiative programs (NRF).

Notes and references

1 For a recent review for ROS/RNS probes, see: X. Chen, X. Tian, I. Shin and J. Yoon, *Chem. Soc. Rev.*, 2011, **40**, 4783–4804.
2 I. Dalle-Donne, R. Rossi, R. Colombo, D. Giustarini and A. Milzani, *Clin. Chem.*, 2006, **52**, 601–623.
3 B. Halliwell and J. M. C. Gutteridge, *Free Radic. Biol. Med.*, Oxford University Press, New York, 2nd edn, 1989.
4 (*a*) K.-I. Setsukinai, Y. Urano, K. Kakinuma, H. J. Majima and T. Nagano, *J. Biol. Chem.*, 2003, **278**, 3170–3175; (*b*) N. Soh, K. Makihara, E. Sakoda and T. Imato, *Chem. Commun.*, 2004, 496–497; (*c*) N. Soh, *Anal. Bioanal. Chem.*, 2006, **386**, 532–543; (*d*) N. Soh, K. Makihara, T. Ariyoshi, D. Seto, T. Maki, H. Nakajima, K. Nakano and T. Imato, *Anal. Sci.*, 2008, **24**, 293–296; (*e*) S. E. Page, K. T. Wilke and V. C. Pierre, *Chem. Commun.*, 2010, **46**, 2423–2425; (*f*) L. Yuan, W. Lin and J. Song, *Chem. Commun.*, 2010, **46**, 7930–7932; (*g*) P. Li, T. Xie, X. Duan, F. Yu, X. Wang and B. Tang, *Chem.–Eur. J.*, 2010, **16**, 1834–1840; (*h*) G. M. Ganea, P. E. Kolic, B. El-Zahab and I. M. Warner, *Anal. Chem.*, 2011, **83**, 2576–2581; (*i*) J.-Y. Kim, W. I. Choi, Y. H. Kim and G. Tae, *J. Controlled Release*, 2011, **156**, 398–405; (*j*) W. T. Huang, W. Y. Xie, Y. Shi, H. Q. Luo and N. B. Li, *J. Mater. Chem.*, 2012, **22**, 1477–1481; (*k*) F. Liu, T. Wu, J. Cao, H. Zhang, M. Hu, S. Sun, F. Song, J. Fan, J. Wang and X. Peng, *Analyst*, 2013, **138**, 775–778.
5 (*a*) N. Soh, K. Makihara, T. Ariyoshi, D. Seto, T. Maki, H. Nakajima, K. Nakano and T. Imato, *Anal. Sci.*, 2008, **24**, 293–296; (*b*) L. Du, N. Ni, M. Li and B. Wang, *Tetrahedron Lett.*, 2010, **51**, 1152–1154; (*c*) M. Yu, G. Zhang, W. Wang, J. Niu and N. Zhang, *Supramol. Chem.*, 2012, **24**, 799–802.
6 N. B. Yapici, S. Jockusch, A. Mosctelli, S. R. Mandalapu, Y. Itagaki, D. K. Bates, S. Wiseman, K. M. Gibson, N. J. Turro and L. Bi, *Org. Lett.*, 2012, **14**, 50–53.
7 (*a*) K. Kundu, S. F. Knight, N. Willett, S. Lee, W. R. Taylor and N. Murthy, *Angew. Chem., Int. Ed.*, 2009, **48**, 299–303; (*b*) P. W. Lin, L. E. S. Myers, L. Ray, S.-C. Song, T. R. Nasr, A. J. Berardinelli, K. Kundu, N. Murthy, J. M. Hansen and A. S. Neish, *Free Radical Biol. Med.*, 2009, **47**, 1205–1211.
8 We have tested several rhodamine cyclic hydrazides prepared from hydrazine (RNHNHR) and rhodamine B and 6G. The probe **1** composed of a cyclic hydrazine and rhodamine 6G exerted best fluorescence properties upon reaction with hydroxyl radical.
9 (*a*) Y.-K. Yang, S. Shim and J. Tae, *Chem. Commun.*, 2010, **46**, 7766–7768; (*b*) H. Kim, S. Lee, J. Lee and J. Tae, *Org. Lett.*, 2010, **12**, 5342–5345; (*c*) H. Kim, K.-S. Moon, S. Shim and J. Tae, *Chem.–Asian J.*, 2011, **6**, 1987–1991; (*d*) S. Shim and J. Tae, *Bull. Korean Chem. Soc.*, 2011, **32**, 2928–2932.
10 (*a*) C. C. Winterbourn, *Free Radical Biol. Med.*, 1987, **3**, 33–39; (*b*) M. Kashima-Tanaka, Y. Tsujimoto and M. Yamazaki, *Int. J. Oral-Med. Sci.*, 2002, **1**, 67–73; (*c*) C.-W. Chen, J.-F. Chiou, C.-H. Tsai, C.-W. Shu, M.-H. Lin, T.-Z. Liu and L.-Y. Tsai, *J. Agric. Food Chem.*, 2006, **54**, 9297–9302.
11 C. Walling, *Acc. Chem. Res.*, 1975, **8**, 125–131.
12 P. K. Chatterjee, S. Cuzzocrea, P. A. J. Brown, K. Zacharowski, K. N. Stewart, H. Mota-Filipe and C. Thiemermann, *Kidney Int.*, 2000, **58**, 658–673.