Title	A modular trigger for the development of selective superoxide probes
Author(s)	Yu, ZH; Chung, CYS; Tang, FK; Brewer, TF; Au Yeung, HY
Citation	Chemical Communications, 2017, v. 53 n. 72, p. 10042-10045
Issued Date	2017
URL	http://hdl.handle.net/10722/248376
Rights	This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License.

Journal Name

COMMUNICATION

A Modular Trigger for the Development of Selective Superoxide Probes

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

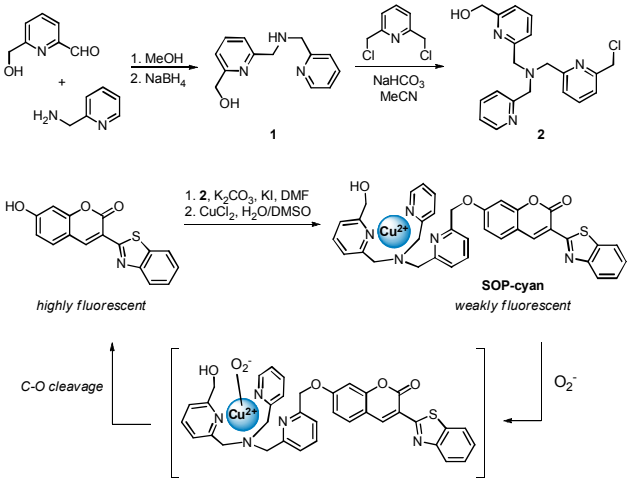
www.rsc.org/

Zuo Hang Yu,[a,†] Clive Yik-Sham Chung,[b,†] Fung Kit Tang,[a,†] Thomas F. Brewer[b] and Ho Yu Au-Yeung[a*]

We report here a new bioinspired copper-based strategy of superoxide sensing and the development of sensitive (>90-fold fluorescence turn-on) and selective superoxide probes for imaging of variations in endogenous superoxide level in various live mammalian cells (HEK293T, HeLa and A431).

Selective recognition of the small, reactive and short-lived superoxide and its detection, in particular in complex biological matrix, are challenging. Intracellularly produced superoxide can lead to oxidative damage of biomolecules and production of other ROS, and its toxicity is implicated in aging[1] and different diseases including Alzheimer's[2] and Parkinson's diseases[3]. Yet, the highly reactive superoxide can also be utilized as an immune response as well as a signaling molecule to mediate cellular processes.[4,5] Bioanalytical tools for selectively recognizing and detecting superoxide from other also highly oxidizing ROS, especially those that can be applied in live biological samples, will therefore be highly valuable for studying the redox biology associated with superoxide. In this regard, fluorescent imaging with superoxide selective probe is a convenient and effective bioanalytical method for the study of the short-lived, highly reactive superoxide in live biological samples.

Currently, there are only few examples of superoxide probes that are applicable for live cell imaging.[6-14] Of these few examples, nucleophilic unmasking and oxidation of organic functional groups on fluorescent dyes by superoxide are the only two strategies of fluorescent superoxide detection. In general, the functional group reacting with superoxide in these probes is directly linked to the fluorophore backbone such that after the superoxide-mediated reaction, the change in the electronic and photophysical properties of the fluorophore results in a fluorescent response. The superoxide sensing chemistry, functional groups and fluorophores in these probes have therefore all to be judiciously chosen in order to obtain the required superoxide reactivity and selectivity. Indeed, different superoxide selectivity over other nucleophilic species has been observed in a series of fluorescein-derived probes based on a nucleophilic substitution mechanism for superoxide sensing.[11] As such, chemical modifications on the probe to tune the photophysical and biological properties may not be trivial and could require highly sophisticated molecular design and heavy synthetic efforts.



Scheme 1. Synthesis of **SOP-cyan** and the proposed reaction of **SOP-cyan** with superoxide.

[a] Department of Chemistry, The University of Hong Kong, Pokfulam Road, Hong Kong, P. R. China.
[b] Department of Chemistry, University of California, Berkeley, California, CA 94720, United States.

† These authors contributed equally.

Electronic Supplementary Information (ESI) available: Experimental details, including synthesis, NMR, LCMS, fluorescence and cell data. See DOI: 10.1039/x0xx00000x

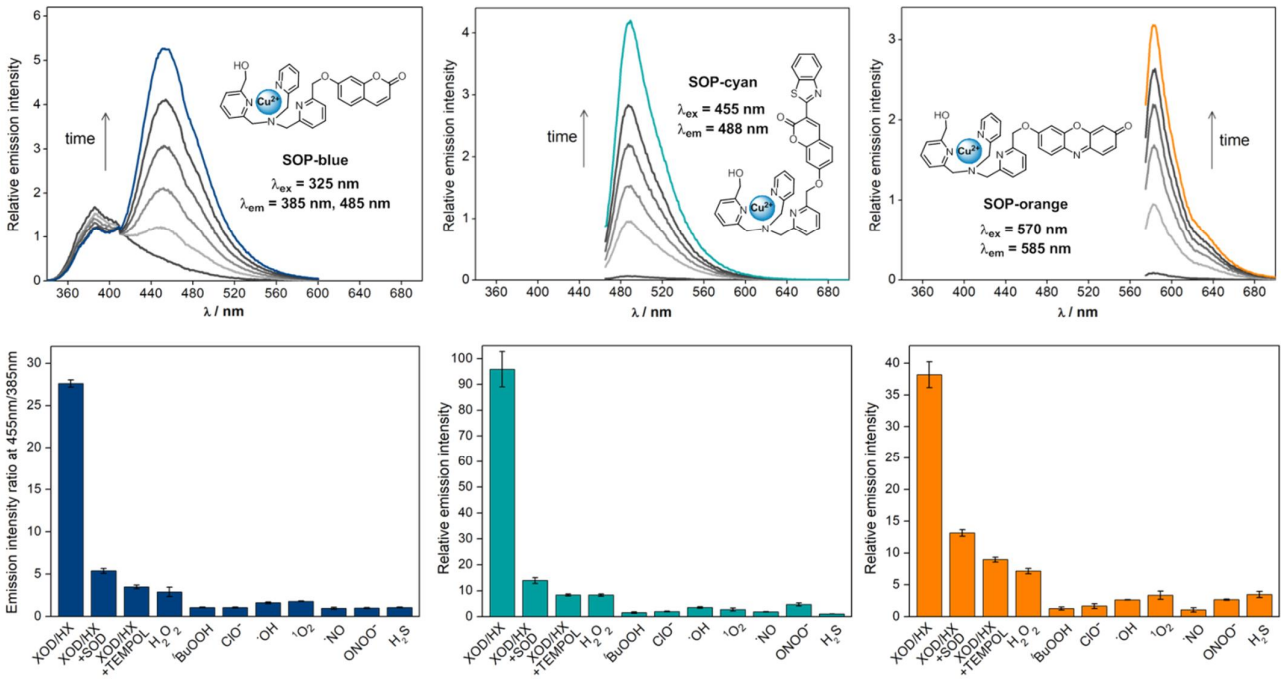


Fig. 1. (a) Time-dependent fluorescence response (0, 3, 5, 7, 10, 60 min) of 5 μM **SOP-blue** (left), **SOP-cyan** (center) and **SOP-orange** (right) towards superoxide (xanthine oxidase: 10 mU/ml; hypoxanthine: 75 μM); and (b) selectivity towards 20 eq. of other reactive species. Error bars are ±SD (n = 3).

Inspired by copper oxygenases which involve a Cu(II)–O_2^- species for substrate oxidation,[15,16] we report here a modular copper-mediated oxidative bond cleavage reaction as a general and versatile strategy for selective superoxide detection. **SOP-cyan**, a Cu(II) complex-caged coumarin, was also developed as a highly sensitive probe for the selective superoxide imaging in various mammalian cell lines. This new metal-based strategy is highly modular that not only different fluorophores can be facilely coupled to the biomimetic complex to obtain superoxide probes of desired photophysical and biological properties, but also the combination of the metal and ligand can be systematically varied to tune the reactivity and selectivity of the probes towards other reactive species.[17-21]

Synthesis of **SOP-cyan** is straightforward with four simple steps as depicted in Scheme 1. **SOP-cyan** consists of a Cu(II) ion coordinated to a hydroxylmethyl substituted tris(2-picolyl)amine ligand, a class of common ligand scaffold for copper-based oxygenase biomimetic, linked to 3-(2-benzothiazolyl)-7-hydroxycoumarin via an C–O bond. The ligand can be easily coupled to other fluorophores via an S_N reaction to obtain probes of desired properties. As caged in its ether form, a 5 μM solution of **SOP-cyan** in Tris buffer (50 mM, pH 7.6) is weakly emissive but showed a strong, 92-fold fluorescence turn-on centered at 488 nm upon reaction with superoxide produced from xanthine oxidase/hypoxanthine within 1 hour (Fig. 1). The fluorescence turn-on was attenuated by competitively removing superoxide by the radical scavenger TEMPOL[22] or superoxide dismutase, confirming that the observed fluorescence responses were superoxide-dependent. In addition, **SOP-cyan** is selective only to superoxide and showed no fluorescence enhancement by other reactive species including H_2O_2, ^{t}BuOOH, $^1O_2$, ·OH, ClO^-, ·NO, $ONOO^-$ and H_2S under the same conditions. The presence of other biological ligands (e.g. histidine, methionine, cysteine, glutathione) and transition metal ions (Fe^{2+}, Fe^{3+} and Zn^{2+}) did not affect the fluorescence response of **SOP-cyan** towards superoxide (Figure S1), further establishing the applicability of the probe in the complex intracellular environment. Taking advantage of the high modularity of our probe design, 7-hydroxycoumarin-based **SOP-blue** and resorufin-based **SOP-orange** have also been synthesized via a straightforward conjugation of the biomimetic complex and the fluorophores (Scheme S1). Similar to **SOP-cyan**, **SOP-blue** and **SOP-orange** also exhibit a selective superoxide fluorescence turn-on, but respectively featuring a blue and orange emission color. **SOP-blue** additionally displays a ratiometric response.[23] Such a tunability of probe properties via only simple synthetic procedures, while at the same time not compromising the reactivity and selectivity of the superoxide trigger, is enabled by this new and modular strategy and is different from existing superoxide sensing methods.

Reactions of **SOP-cyan** with superoxide and other reactive species were further studied by LCMS. First, it was found that **SOP-cyan** was stable and no significant difference was noticed in the chromatograms of the same **SOP-cyan** solution analyzed over three days (Fig. S2). The good stability of **SOP-cyan** is probably due to the polydentate nature of the ligand. Next, the expected oxidative C–O bond cleavage has been confirmed by the appearance of a new peak corresponding to the

uncaged 3-(2-benzothiazolyl)-7-hydroxycoumarin (*m/z* = 296.1 $[M+H]^+$) in the chromatogram of the reaction mixture of **SOP-cyan** and superoxide (Fig. S5). In addition, LCMS studies showed that reactions of **SOP-cyan** with H_2O_2, ^tBuOOH, ClO^-, ·OH, and $^1O_2$ and $ONOO^-$ resulted in no oxidative bond cleavage (Fig. S8). Some degree of Cu(II) displacement by excess NO or H_2S was observed, yet the resulting ligand-fluorophore conjugate was still caged in its non-emissive form and thus producing no fluorescence response. Notably, such competitive Cu(II) displacement is a common strategy in the design of fluorescent probes for NO, H_2S and related small reactive species, in which a fluorescent enhancement is a result of the removal of the paramagnetic transition metal quencher.[24-27] In the present case of **SOP-cyan**, the fluorophore is still covalently caged in its non-emissive form even when the Cu(II) is displaced, and therefore distinguishing **SOP-cyan** with a unique mechanism of superoxide selectivity. Finally, reactions of **SOP-cyan** with superoxide at different pH (pH 4.5 to pH 8.5) all showed the release of the fluorophore (Fig. S11), suggesting that this detection strategy could be applied to prepare superoxide probes that are applicable at different intracellular pH.

After demonstrating the *in vitro* analytical performance of **SOP-cyan**, we next sought to apply the probe in the fluorescent imaging of superoxide in living cells under biologically relevant conditions. Menadione (vitamin K3) stimulation of human cervical carcinoma (HeLa) and human embryonic kidney (HEK293T) cells was first studied as models of stimulated superoxide generation and initiation of ROS-mediated signaling upon intracellular activation of the vitamin.[28,29] As shown in Fig. 2 and S11, HeLa cells stimulated by menadione for 30 min and stained by **SOP-cyan** exhibited a marked 2.2-fold increase in intracellular fluorescence when compared to control cells only stained by the probe. Similar increase in the intracellular fluorescence in menadione-stimulated cells was also observed from HEK293T cells stained by the probe under similar conditions (Fig. S12). In addition, a significantly reduced intracellular fluorescence was observed in both HeLa and HEK293T cells pretreated with the cell-permeable superoxide dismutase PEG-SOD (40 U/ml) for 24 hours when compared to control cells, showing that **SOP-cyan** is also capable of reporting decreases in the endogenous superoxide level in these cell lines. Nuclear staining and MTT assays showed that **SOP-cyan** is not cytotoxic and the cells were viable throughout the experiment (Fig. S11, S12 and S15),[30] showing that the probe is applicable in the imaging of both stimulated production and changes in endogenous level of intracellular superoxide in different cells. In addition to vitamin K stimulation, imaging of NADPH oxidase (NOX)-mediated superoxide production and ROS signaling upon binding of epidermal growth factor (EGF) to epidermal growth factor receptor (EGFR) by **SOP-cyan** was also studied.[31-33] As shown in Fig. 2 and S13, epidermoid carcinoma cells A431, which expresses a high level of EGFR, treated with EGF (100 ng/ml) exhibited an increase in the intracellular fluorescence upon staining by **SOP-cyan**. On the other hand, A431 cells treated with 5 μM diphenyleneiodonium (DPI), an NOX inhibitor, showed a significant decrease in the intracellular fluorescence, suggesting that **SOP-cyan** is sensitive to image both the upregulation and downregulation of NOX-mediated superoxide signaling. Taken together, these experiments have successfully established the applicability of **SOP-cyan** in detecting changes of superoxide level under biologically relevant conditions in different cell lines, and show the potential of the probe as an efficient imaging tool for studying the redox biology of superoxide in a cellular setting.

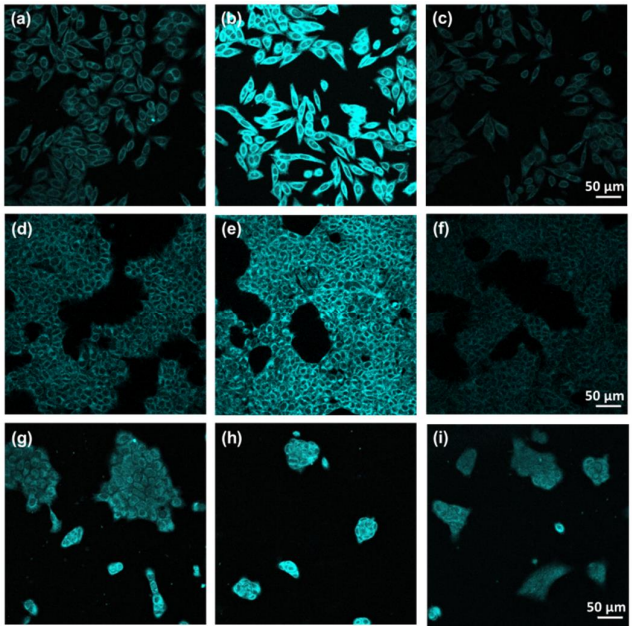


Fig. 2. Representative confocal microscopy images of (a-c) HeLa, (d-f) HEK293T and (g-i) A431 cells stained with 5 μM **SOP-cyan** for 30 min. (a, d and g) Control cells. (b, e) Cells treated with 10 μM menadione for 30 min. (c, f) Cells treated with PEG-SOD (40 U/ml) for 24 hours. (h) Cells treated with EGF (100 ng/ml) for 4.5 hours. (i) Cells treated with 5 μM DPI for 4.5 hours. Scale bar = 50 μm.

In summary, we have described a versatile reaction-based strategy for the selective detection of superoxide using a bioinspired copper(II) complex, and its development into three highly sensitive and selective superoxide probes with various emission colors. Live cell imaging studies showed that **SOP-cyan** is capable of detecting changes in endogenous superoxide levels in different cell types (HeLa, HEK293T and A431) under relevant biological conditions. This new superoxide sensing strategy features a modular bond cleavage mechanism for fluorescence turn-on which not only allows straightforward chemical modifications of the photophysical and/or cellular properties of the probe via simple conjugation of the trigger with different fluorophores or reporter groups, but also provides a unique mechanism for attaining superoxide reactivity and selectivity that distinguishes **SOP-cyan** from existing superoxide probes. In addition, systematic variations of the coordination environment of the biomimetic complex to change the chemical reactivity of the trigger for the selective

recognition and detection of other reactive species and small molecules, and applications of the biomimetic trigger in prodrugs, controlled-release and other responsive materials can also be expected.

This work was supported by the Croucher Foundation and the Research Grants Council of the Hong Kong Special Administrative Region, China (HKU 106150183). Z. H. Yu and F. K. Tang are recipients of the Postgraduate Scholarship from The University of Hong Kong. C. Y.-S. Chung thanks the Croucher Foundation for the Croucher Foundation Fellowship and T. F. Brewer was supported by the T23 Chem Bio Training Grant. We thank Prof. Vivian W.-W. Yam for allowing to access to their fluorescence spectrophotometer, Dr. Margaret C.-L. Yeung for assisting in the collection of some of the emission data and Prof. Christopher J. Chang for his helpful support in this project. We acknowledge UGC funding administered by The University of Hong Kong for supporting the Electrospray Ionization Quadrupole Time-of-Flight Mass Spectrometry Facilities under the Support for Interdisciplinary Research in Chemical Science and the technical assistance from Alison Killilea and Carissa Tasto (UC Berkeley Tissue Culture Facility) for cell culture experiments.

Notes and references

1 S. I. Liochev *Free Radic. Biol. Med.,* 2013, **60**, 1.
2 C. A. Massaad, T. M. Washington, R. G. Pautler, E. Klann *Proc. Natl. Acad. Sci. USA,* 2009, **106**, 13576.
3 M. Vila, S. Przedborski *Nat. Rev. Neurosci.,* 2003, **4**, 1.
4 D. Basudhar, L. A. Ridnour, R. Cheng, A. H. Kesarwala, J. Heinecke, D. A. Wink *Coord. Chem. Rev.,* 2016, **306**, 708.
5 T. M. Buetler, A. Krauskopf, U. T. Ruegg *News Physiol. Sci.,* 2004, **19**, 120.
6 A. Gomes, E. Fernandes, J. L. F. C. Lima *J. Biochem. Biophys. Methods,* 2005, **65**, 45.
7 K. M. Robinson, M. S. Janes, M. Pehar, J. S. Monette, M. F. Ross, T. M. Hagen, M. P. Murphy, J. S. Beckman *Proc. Natl. Acad. Sci. USA,* 2006, **103**, 15038.
8 S. T. Manjare, S. Kim, W. D. Heo, D. G. Churchill *Org. Lett.,* 2014, **16**, 410.
9 W. Zhang, P. Li, F. Yang, X. Hu, C. Sun, W. Zhang, D. Chen, B. Tang *J. Am. Chem. Soc.,* 2013, **135**, 14965.
10 P. Li, W. Zhang, K. Li, X. Liu, H. Xian, W. Zhang, B. Tang *Anal. Chem.,* 2013, **85**, 9877.
11 H. Maeda, K. Yamamoto, Y. Nomura, I. Kohno, L. Hafsi, N. Ueda, S. Yoshida, M. Fukuda, Y. Fukuyasu, Y. Yamauchi, N. Itoh *J. Am. Chem. Soc.,* 2005, **127**, 68.
12 J. J. Hu, N.-K. Wong, S. Ye, X. Chen, M.-Y. Lu, A. Q. Zhan, Y. Guo, A. C.-H. Ma, A. Y.-H. Leung, J. Shen, D. Yang *J. Am. Chem. Soc.,* 2015, **137**, 6837.
13 J. Zhang, C. Li, R. Zhang, F. Zhang, W. Liu, X. Liu, S. M.-Y. Lee, H. Zhang, *Chem. Commun.,* 2016, **52**, 2679.
14 X. Gao, G. Feng, P. N. Manghnani, F. Hu, N. Jiang, J. Liu, B. Liu, J. Z. Sun, B. Z. Tang *Chem. Commun.* 2017, **53**, 1653.
15 S. T. Prigge, R. E. Mains, B. A. Eipper, L. M. Amzel *Cell Mol. Life Sci.,* 2000, **57**, 1236.
16 S. T. Prigge, B. A. Eipper, R. E. Mains, L. M. Amzel *Science,* 2004, **304**, 864.
17 H. Y. Au-Yeung, J. Chan, T. Chantarojsiri, C. J. Chang *J. Am. Chem. Soc.,* 2013, **135**, 15165.
18 H. Y. Au-Yeung, E. J. New, C. J. Chang *Chem. Commun.,* 2012, **48**, 5268.
19 M. Taki, S. Iyoshi, A. Ojida, I. Hamachi, Y. Yamamoto, *J. Am. Chem. Soc.,* 2010, **132**, 5938.
20 M. Taki, K. Akaoka, K. Mitsui, Y. Yamamoto *Org. Biomol. Chem.,* 2014, **12**, 4999.
21 M. C. Heffern, H. M. Park, H. Y. Au-Yeung, G. C. Van de Bittner, C. M. Ackerman, A. Stahl, C. J. Chang *Proc. Natl. Acad. Sci.,* 2016, **113**, 14219.
22 C. S. Wilcox *Pharmacol. Ther.,* 2010, **126**, 119.
23 A ratiometric response is an advantageous probe feature that allows self-referencing for correcting artefacts in the staining of biological samples.
24 M. H. Lim, D. Xu, S. J. Lippard *Nat. Chem. Biol.,* 2006, **2**, 375.
25 X. Hu, J. Wang, X. Zhu, D. Dong, X. Zhang, S. Wu, C. Duan *Chem. Commun.,* 2011, **47**, 11507.
26 M. G. Choi, S. Cha, H. Lee, H. L. Jeon, S.-K. Chang *Chem. Commun.,* 2009, **47**, 7390.
27 K. Sasakura, K. Hanaoka, N. Shibuya, Y. Mikami, Y. Kimura, T. Komatsu, T. Ueno, T. Terai, H. Kimura, T. Nagano *J. Am. Chem. Soc.,* 2011, **133**, 18003.
28 G. Loor, J. Kondapalli, J. M. Schriewer, N. S. Chandel, T. L. Vanden Hoek, P. T. Schumacker *Free Radic. Biol. Med.,* 2010, **49**, 1925.
29 W. Lee, M. Xu, Y. Li, Y. Gu, J. Chen, D. Wong, P. C. Fung, J. Shen *Arch. Biochem. Biophys.,* 2011, **514**, 57.
30 Co-staining studies in HEK293T cells with organelle-specific probes showed that **SOP-cyan** localizes in the ER (Fig. S16).
31 Y. S. Bae, S. W. Kang, M. S. Seo, I. C. Baines, E. Tekle, P. B. Chock, S. G. Rhee *J. Biol. Chem.,* 1997, **272**, 217.
32 T. W. Cunningham, D. Kuppuswamy, L. J. Pike *J. Biol. Chem.,* 1989, **264**, 15351.
33 D. I. Brown, K. K. Griendling *Free Radic. Biol. Med.,* 2009, **47**, 1239.

