Fluorescein-Containing Superoxide Probes with a Modular Copper-Based Trigger

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Dedication ((optional))

Abstract: Fluorescein-derived superoxide probes featuring a copper-mediated oxidative ether bond cleavage for superoxide imaging in live cells are described. These probes feature a copper(II) complex that can be activated by superoxide to effect an ether bond cleavage to uncage a fluorescein reporter. Compared to other superoxide sensing moiety, this bond cleavage strategy can be modularly adapted to fluorescent reporters of different properties without compromising the superoxide reactivity and selectivity. The green emitting SOP-green and its lysosome-targeting analogue Lyso-SOP-green have been successfully developed. Both probes are sensitive with more than 30-fold fluorescence enhancement towards superoxide and are highly selective with no significant response towards other reactive oxygen species. A structure-activity relationship study of the copper-based superoxide trigger showed that the secondary coordination environment of the copper(II) center is important for the superoxide reactivity and selectivity. The probes have been applied in imaging changes in intracellular superoxide level in live HeLa and HEK293T cells upon menadione stimulation and also in a cellular inflammation model in RAW 264.7 cells.

Introduction

Superoxide is a reactive oxygen species (ROS) that on one hand upon overproduction in cells will lead to oxidative stress and is implicated in diseases such as Parkinson disease, cancer, and aging,[1] and on the other hand is enzymatically produced for cellular defense, signalling, regulation of gene expression, and other important biochemical processes.[2] Levels of superoxide in cells are tightly regulated and its selective detection in the complex biological matrix is therefore highly important for studying the redox biology of this short-lived, reactive species. In particular, fluorescent imaging using superoxide probes is an attractive bioanalytical method for studying the ROS because of various advantages including the applicability in different biological samples, non-destructiveness, ease of use, and possibility of multi-analyte detection using probes in different emission channels.[3]

Developing a selective trigger that is responsive to superoxide and is able to produce a detectable photophysical output is one major challenge in the design of effective fluorescent superoxide probes. For the oxidizing and nucleophilic reactivity of superoxide, direct oxidation[4] and nucleophilic substitution[5] that respectively changes the electronic properties of the fluorophore and displaces an emission quencher by superoxide are currently the two main strategies in developing superoxide responsive triggers. These two strategies may however not be compatible with chemical modifications as these superoxide triggers are often electronically conjugated to the fluorophore. A change in the fluorophore covalent structure is often associated with a change in the superoxide reactivity and selectivity, and therefore tuning the photophysical, analytical and biological properties of the probes may not be straightforward.

One other well-studied chemistry of superoxide is its reactivity with transition metals. Superoxo species of iron, copper, manganese and other metals have been identified as important intermediates for different metalloproteins and metalloenzymes.[6] Inspired by the role of Cu(II) superoxo species that is involved in substrate oxidation by copper oxygenases, we have previously communicated the use of a Cu(II) complex supported by the tetradentate tris(2-pyridylamine) ligand with a hydroxyl pendant as a superoxide responsive trigger for the oxidative release of coumarin-based fluorophores.[7] These blue and cyan emitting coumarin-based probes are highly selective towards superoxide against other ROS including hydrogen peroxide, hydroxyl radical and hypochlorite, and are applicable in live cell imaging. In this work, we further extend our bond cleavage strategy to develop fluorescein-based superoxide probes by modularly linking the superoxide responsive Cu(II) complex to fluorescein-derived fluorophores. Not only the excitation and emission wavelengths of the superoxide probes are red-shifted to match better with common instrumental setups, additional organelle targeting groups can also be attached without compromising the superoxide reactivity and selectivity. Here, the development of the green emitting SOP-green and the lysosome targeting Lyso-SOP-green, and their applications in imaging changes in intracellular superoxide level are presented. The copper-mediated ether bond cleavage and its structure-activity relationship have also been carefully characterized and studied.

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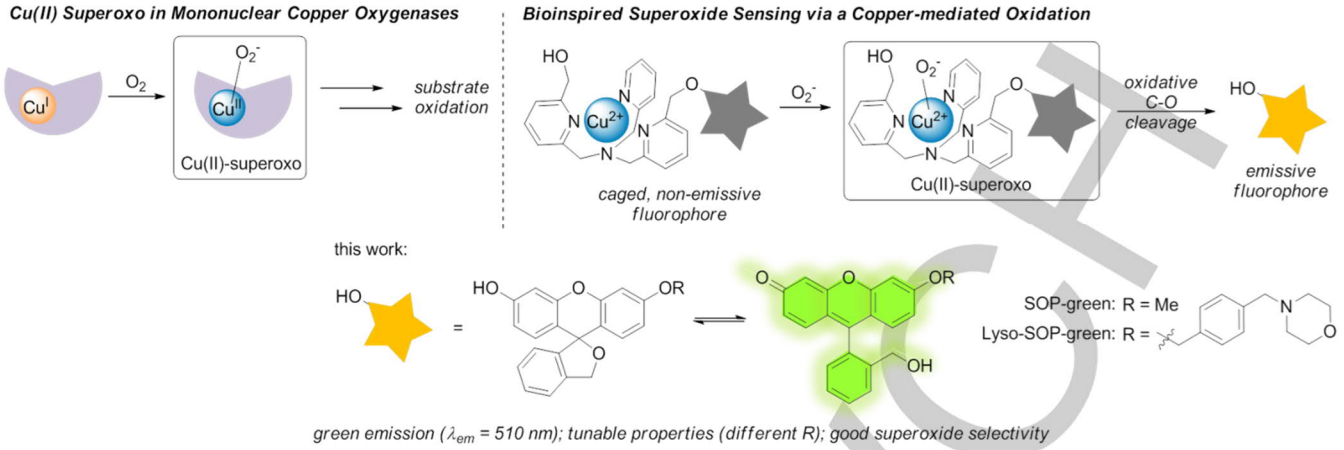

Scheme 1. A copper(II)-superoxo species generated from oxygenation of Cu(I) involved in substrate oxidation by copper oxygenases (left) inspires the design of a new copper-based superoxide detection (right). A fluorescein reporter that is originally caged in its non-emissive form will be released and produce a fluorescent signal upon a reaction of the Cu(II) with superoxide that leads to an oxidative ether bond cleavage.

Results and Discussion

Fluorescein is of the one most commonly used fluorophores in fluorescence microscopy and related bioanalytical techniques because of its various favorable properties such as strong absorption, high quantum yield, ease of functionalization and biocompatibility.[8] New superoxide probes based on fluorescein-derived fluorophores are therefore anticipated to be more versatile and compatible with existing microscopic techniques and instrumentations. Synthesis of SOP-green is straightforward as shown in Scheme S1. Upon caging the fluorescein fluorophore as an ether of the tripodal ligand, conjugation of the xanthene moiety is disrupted and the compound becomes non-emissive.[9] A superoxide-induced ether bond cleavage and a keto-enol tautomerization of the released phenol will ring-open the spiroether, restore the conjugation and produce an emission enhancement. As expected, a 5 μM solution of SOP-green in Tris buffer (50 mM, pH 7.6) is weakly emissive. Upon treatment with superoxide generated from xanthine oxidase (XOD, 10 mU/ml) and hypoxanthine (HX, 75 μM), a strong emission centered at 510 nm (λ_{ex} = 470 nm) was observed with a 33-fold fluorescence enhancement after a 1-hour reaction (Figure 1). Significantly reduced fluorescent responses were observed when the reaction between SOP-green and superoxide was repeated in the presence of a radical scavenger (4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl, TEMPOL) or superoxide dismutase that rapidly remove superoxide. Furthermore, the fluorescence response of SOP-green is highly selective and other reactive species including H_2O_2, ^tBuOOH, ClO^-, •OH, $^1O_2$, •NO, $ONOO^-$ and H_2S gave only negligible fluorescence responses. The presence of biologically relevant transition metal ions such as Zn^{2+}, Fe^{2+} and Fe^{3+} also did not affect the superoxide response (Figure S4). The superoxide-induced ether bond cleavage of SOP-green has been confirmed by LCMS. After a 1-hour reaction, a peak corresponding to the released hydroxylmethylfluorescein (retention time = 21.0 min, *m/z* = 333.0 $[M+H]^+$) was identified from the LC chromatogram of the reaction mixture with a cleavage yield of about 24%. Other oxidation products were also observed (Figure S2). In contrast, no uncaged fluorophore was observed from the reaction of SOP-green and other studied reactive species, reinforcing the notion that the probe is highly selective towards superoxide. Moreover, the inability of these ROS to effect the ether bond cleavage is consistent with a copper-based oxidation via an active oxidant generated from the reaction of superoxide with the Cu(II).

By further attaching a morpholine containing moiety as a lysosome targeting group,[10] Lyso-SOP-green was obtained. Fluorescence studies showed that the superoxide selectivity of Lyso-SOP-green are very similar to those of SOP-green, demonstrating that this bond cleavage-based strategy could be versatilely applied to other superoxide-triggered release. Despite both SOP-green and Lyso-SOP-green contain the same fluorescein reporter and the same copper(II)-based superoxide trigger, the fluorescence response rate and the fluorescence intensity seems to be also dependent on the chemical properties of the other ether group (i.e. R in Scheme 1), suggesting that the organelle-targeting group may also affect other properties of the probes. In particular, preliminary studies showed that the fluorescence quantum yield of the fluorescein can also be tuned by using different R groups. Chemical properties of the R group such as acid-base properties, charge and solubility may also affect the reaction between the probe and superoxide, and lead to a different response rate of the probes. Further studies will be needed to elucidate these effects. Nevertheless, the selective superoxide response of the copper(II) complex has been successfully applied on the fluroescein fluorophore, which may also be extended to other fluorescent reporters to give probes of other photophysical and biological properties.

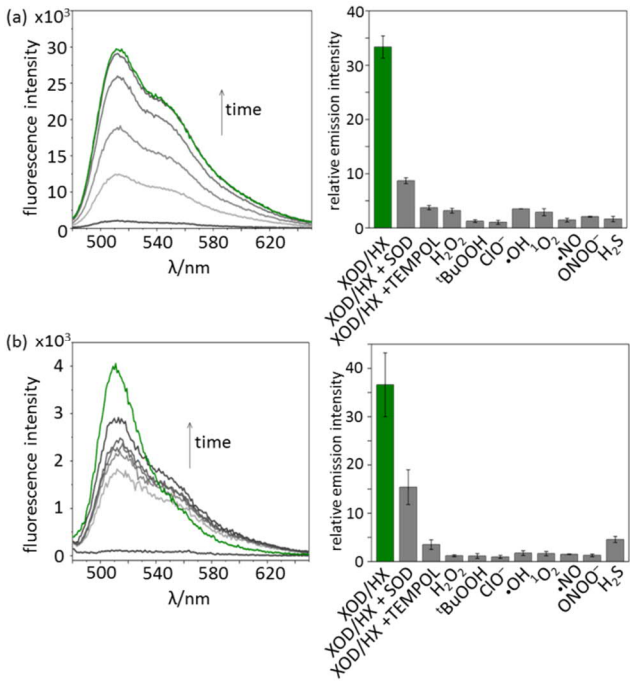

Figure 1. Time-dependent fluorescence response of 5 μM (a) SOP-green (0, 3, 5, 7, 10, 60 min) and (b) Lyso-SOP-green (0, 10, 20, 30, 40, 50, 60 min) towards superoxide (XOD: 10 mU/mL; HX: 75 μM) and 20 eq. of other reactive species. Error bars are ±SD (n = 3).

To further understand the ether bond cleavage, the Cu(II) coordination environment was studied by single crystal X-ray diffraction of a model Cu(II) complex (Figure 2a). The Cu(II) center was found to be 5-coordinated in a square pyramidal geometry (τ_5 = 0.11) with four nitrogen donors from the ligand and a chloride in the primary coordination sphere. The chloride is suggested not to be involved in the oxidative bond cleavage as samples of SOP-green prepared from other Cu(II) salts such as perchlorate did not show significantly different superoxide responses (Figure S4). No close contact between the metal and the hydroxylmethyl group was identified. The non-coordinating nature of the hydroxylmethyl group has also been reported in related copper(II) complexes.[11] Interestingly, the Cu(II) complex-fluorophore conjugate **1** with an unsubstituted tris(2-pyridyl)amine ligand was found to be also reactive towards hydroxyl radical and produced a fluorescence turn-on that is comparable to that of superoxide. A preliminary structure-activity study by replacing the hydroxylmethyl substituent to other functional groups resulted in very different superoxide reactivity and selectivity patterns, showing that the secondary coordination environment is important to the reactivity of the Cu(II)-bound compounds towards different reactive species (Figure 2c). Indeed, significant effects of steric protection, additional weak interactions (e.g. hydrogen bonding) and possible proton transfer from functional groups in the secondary coordination sphere on the stability of the active copper-oxygen oxidant and the oxidative reactivity of copper oxygenases and related synthetic models have also been recognized and reported,[12] suggesting that the observed superoxide reactivity and selectivity is a result of the correct combination of both the primary and secondary coordination environments of the copper. In copper oxygenases and related synthetic models, hydrogen atom abstraction by a copper-oxygen species has been identified as a key step in substrate oxidation.[13] Computational studies on our system also suggest that a similar mechanism could be viable, with the hydrogen atom abstraction by the Cu(II) superoxo species being the rate determining step with an activation energy of about 19 kcal/mol (Figure S6).

Applicability of SOP-green in imaging intracellularly produced superoxide was evaluated in live human embryonic kidney HEK293T cells. In particular, intracellular superoxide production was stimulated by treating the cells with menadione (vitamin K3) which is known to induce intracellular superoxide production through a quinone/hydroquinone redox recycling.[14] As shown in Figure 3, menadione-stimulated cells yielded a significant fluorescence enhancement upon staining by SOP-green as compared to control cells, although the probe may not be sensitive enough for imaging superoxide at an endogenous level (Figure S7). A similar increase in intracellular fluorescence from menadione treatment was also observed from human cervical carcinoma HeLa cells, suggesting the probes can be used in different cell types (Figure S8). Co-staining experiments showed that SOP-green may have different localization properties in different cell types (Figures S9 and S10). Furthermore, nuclear staining experiments and MTT assays revealed that the cells are viable throughout the experiments and that the probes are non-cytotoxic at the working conditions (Figure S11).

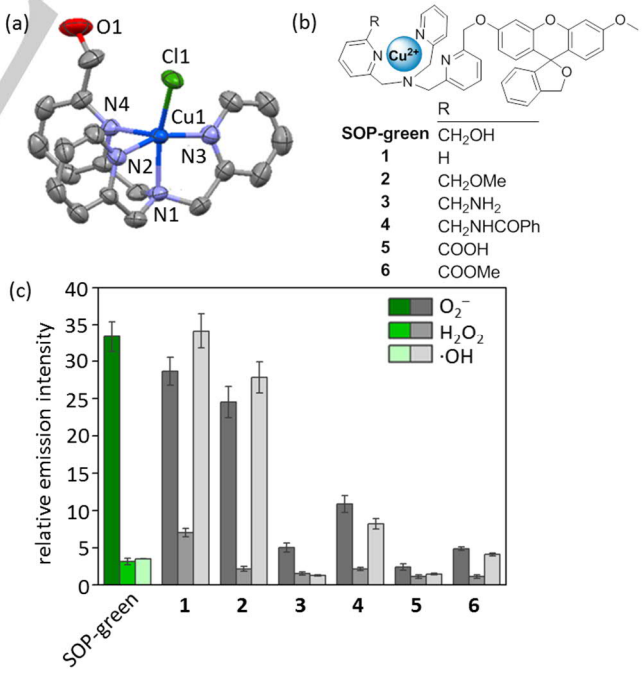

Figure 2. (a) X-ray structure of the cation of $[Cu(\mathbf{L})]_2[CuCl_4]$ (L = a truncated model ligand) at 50% thermal ellipsoid; and (b) molecular structures of SOP-green derivatives with different substituents and (c) their fluorescence

responses towards $O_2^{\cdot-}$. H_2O_2 and •OH. Only SOP-green shows good superoxide reactivity and selectivity. Error bars are ±SD (n = 3). CCDC 1556895 contains the supplementary crystallographic data for this paper.

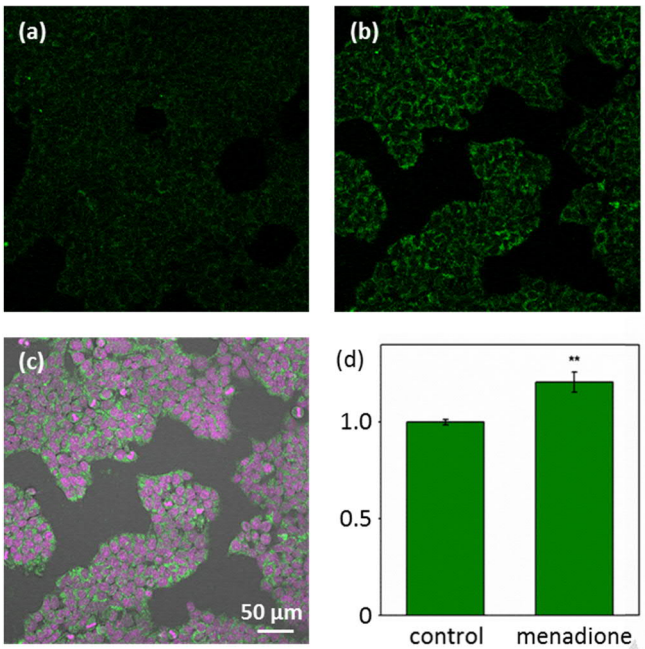

Figure 3. Representative confocal microscopy images of HEK293T cells stained with 5 µM SOP-green for 30 min. (a) Control cells; (b) cells pretreated with 10 µM menadione for 30 min; (c) overlays of bright field, SOP-green in (b) and far-red nuclear stain DRAQ5™ images; and (d) average fluorescence intensity of SOP-green from cells treated under the conditions in (a) and (b) from triplicate experiments. Error bars are ±SD (n = 3). Statistical analysis was performed with a two-tailed Student's *t*-test. **: $p < 0.01$.

The use of Lyso-SOP-green for visualizing changes in superoxide level in lysosome was also studied. In cells, lysosome is an organelle involving biomolecule degradation, signaling, energy metabolism and other cellular activities.[15] Recent studies have suggested that lysosomal activities could be regulated by ROS,[16] and a probe that specifically reports changes in lysosomal superoxide level will be an important imaging tool for studying related cellular processes. The well-studied inflammation model using RAW 264.7 cells was applied for inducing lysosomal superoxide production upon lipopolysaccharide (LPS) stimulation.[17] The lysosome targeting ability of Lyso-SOP-green was first evaluated by a co-localization study with the commercially available LysoTracker Red. As shown in Figure 4, a good overlap of fluorescence signals from Lyso-SOP-green and LysoTracker Red was observed with a Pearson's correlation coefficient of 0.84, demonstrating a good lysosome targeting ability of the probe (Figure 4). More importantly, a significant increase in intracellular fluorescence was observed in cells that are stimulated by LPS (1000 ng/mL) as compared to that of control cells, consistent with the expected increase in superoxide production by NOX proteins upon LPS challenge (Figure 5).[16]

Furthermore, the LPS-induced intracellular fluorescence could be attenuated by treating the cells with the NOX inhibitor apocyanin (300 µM)[18] or Ginsenoside Rb1 (1 µg/mL)[19] which is a natural product with anti-oxidizing properties. All together, these results demonstrate the applicability of Lyso-SOP-green as an imaging probe for visualizing superoxide in lysosome.

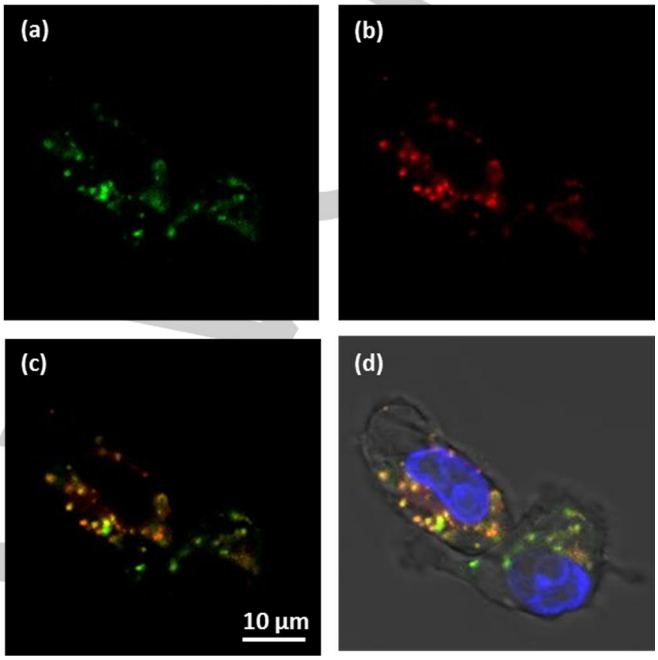

Figure 4. Representative confocal microscopy images of RAW 264.7 stained by (a) Lyso-SOP-green; (b) LysoTracker Red; (c) overlay of the two images in (a) and (b); and (d) overlaid of the bright field image and the image in (c) with Hoechst stain.

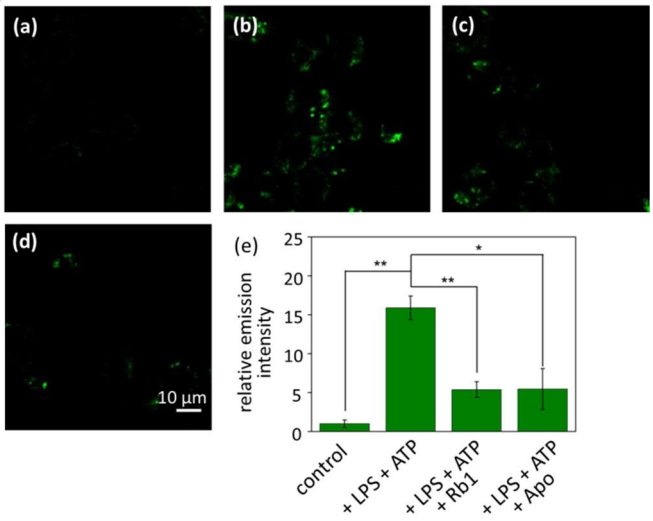

Figure 5. Representative confocal microscopy images of RAW 264.7 cells stained with 10 µM Lyso-SOP-green for 30 min. (a) Control cells; (b) cells treated with LPS (1000 ng/mL) for 15 hours; (c) cells treated with LPS (1000 ng/mL) and Ginsenoside Rb1 (1 µg/mL) for 15 hours; (d) cells treated with

LPS (1000 ng/mL) and apocyanin (300 μM) for 15 hours. (e) Average fluorescence intensity of Lyso-SOP-green in RAW 264.7 cells treated under the conditions in (a)–(d) from triplicate experiments. Error bars are ±SD (n = 3). Statistical analysis was performed with a two-tailed Student's *t*-test. *: $p<0.05$; **: $p<0.01$.

Conclusions

In summary, development and applications of SOP-green and Lyso-SOP-green in live cell imaging have been described. One key feature of these probes is the copper-mediated oxidative cleavage that is selectively activated by superoxide. Because of the modular nature of the bond cleavage-based superoxide sensing strategy, the photophysical, bioanalytical and other properties can be tuned straightforwardly without affecting the sensitivity and selectivity of the probe. SOP-green and Lyso-SOP-green have been successfully developed from fluorescein which is one of the most commonly used fluorophores in fluorescence imaging, flow cytometry and other fluorescence-based bioanalytical techniques. A simple structure-activity relationship study suggests that the environment of the secondary coordination sphere is a determining factor for the reactivity of the copper(II) towards different reactive species. Computational studies suggested that a hydrogen atom abstraction could be involved in the superoxide-induced bond cleavage. Further studies on the copper reactivity and exploitation of the metal-based bond cleavage as a new recognition strategy for reactive species and small molecules that are otherwise difficult to be detected by host-guest chemistry are currently underway in our laboratory.

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Keywords: copper • fluorescence • oxidation • reactive oxygen species • superoxide

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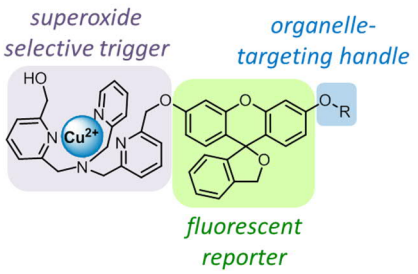
Development of fluorescein-based superoxide probes via a modular approach is described. A copper(II) complex has been exploited as a selective superoxide trigger for the release of an emissive fluorophore. Reactivity of the copper(II) complex is found to be dependent on both the primary and secondary coordination environment and the probes have been applied in the imaging of intracellular superoxide.



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Fluorescein-Containing Superoxide Probes with a Modular Copper-Based Trigger