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Mitochondrion Targeting Fluorescent Probe for Imaging of Intracellular Superoxide Radical

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An amine-reactive fluorogenic molecule specifically turned on by superoxide radicals (O$_2^-$) was synthesized and coupled to a mitochondrial (MT) targeting peptide. The obtained probe showed superior uptake and MT targeting capabilities; and successfully detected the change of O$_2^-$ levels in cells treated by chemical stimuli or single-walled carbon nanotubes.

Biological systems continuously produce free radicals via a wide range of physiological processes. The radicals can act as secondary messengers and control diverse activities like host defense, inflammation, and cellular signaling. The levels of radicals are tightly controlled by a series of antioxidants and enzymes. If the balance is broken due to malfunction of the antioxidant protection systems, damages including aging and pathological conditions like cancer, cardiovascular, inflammatory and degenerative diseases, could occur. Still, not enough is known about the mechanisms of oxidant and antioxidant action so that effective interventions can be designed, calling for more powerful methodologies to be developed for detecting oxidant production specifically and quantitatively.

Generally produced from oxygen reduction by electrons leaked out from the mitochondrial respiratory chains, superoxide radical (O$_2^-$) is the common source of most of the reactive oxygen or nitrogen species (ROS or RNS) found in cells. It can go through dismutation and form H$_2$O$_2$ or be oxidized by NO and generate ONOO$^{-}$. These products then continue to react and produce a series of ROS and RNS. Such an essential role makes it imperative to assess O$_2^-$ generation in mechanistic studies on oxidative damage and antioxidant protection.

Current tools for O$_2^-$ assessment often fall in three categories: detection of the unpaired electron by electron spin resonance (EPR); electrochemical sensors based on its redox property; and optical methods that permits high-throughput screening and imaging in live cells and animals. They can effectively detect the presence of superoxide radicals, but still have limitations. For example, spin traps have been developed to detect superoxide and hydroxyl radical by EPR, but they are only suitable for measurement of extracellular radicals. Similarly, electrochemical sensors utilizing immobilized enzymes, nanoparticles, or microelectrodes, have been fabricated for detection of superoxide released by cells or in solutions. Electrodes with immobilized enzymes can provide good specificity but are complicated and delicate to fabricate. Because of their measurement simplicity and capability to enable *in vitro* and even *in vivo* imaging, optical methods have been widely used, taking advantages of various fluorescence and chemiluminescent probes. They offer good sensitivity, either by the dye itself or by nanoparticle-assisted signal enhancement; and can dynamically image O$_2^-$ fluctuation using the two-photon technology. However, specificity is a big concern. The cyanine-based sensors simultaneously detect other ROS like hydroxyl radical together with superoxide. Hydroethidine (HE), the most common fluorescent sensor for O$_2^-$, can be oxidized by other intracellular processes involving peroxidase and cytochromes to more than one red fluorescence products. Specificity of the HE-based fluorescence assay can be enhanced by HPLC separation of the multiple products, or by using two different excitation wavelengths. Similar cross-reaction problems could exist for other fluorescent probes that sense superoxide radicals based on redox reactions.

Besides high sensitivity and specificity, imaging probes should target specific organelles, because superoxide radicals are typically generated in mitochondria via respiration, in phagosomes by NADPH oxidase for defense of invading pathogens, or in endoplasmic reticulum during process of protein synthesis.

**Figure 1.** a) Synthesis process of probe 1. The sulfanyl groups could be nucleophilic substituted by O$_2^-$ to emit strong fluorescence. The b) absorption and c) fluorescence emission (λ$_{ex}$=495 nm) spectra of 32 μM 1 before and after reaction with 100 μM K$_3$O$_2$ in 50 mM phosphate buffer (pH 7.4). The spectra of FITC were also displayed.

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Their anionic nature makes them impermeable through the organelle membranes and they are converted at their production sites by enzymes or other processes. Monitoring their production and conversion for mechanistic study thus requires probes that can specifically locate at the production sites to gain more information.

A group of fluorescent probes that detect superoxide via a nonredox mechanism have been reported by Itoh et al.\textsuperscript{36,37} Superoxide reacts with this group of bis(2,4-dinitrobenzenesulfonyl) fluorescein molecules as a nucleophile, instead of as a reductant. The reaction causes the cleavage of the benzensulfonyl groups, turning on the fluorescence of fluorescein. Similar reaction mechanism was also employed to design the turn-on $O_2^-$ sensor molecule, bis(diphenylphosphinyl) fluorescein.\textsuperscript{38} However, localized detection of endogenous $O_2^-$ has not been demonstrated with such dyes. Following the same synthetic route discovered by the Itoh group, we prepared the bi-substituted Bis(2,4-dinitrobenzenesulfonyl) fluorescein isothiocyanate (1) (Figure 1a). The isothiocyanate (-SCN) group is not only readily reactive to free amines that enables easy conjugation to biological molecules, but also enhances the probe’s specificity to $O_2^-$. Conjugated to the mitochondrial locating signal (MLS) peptide (NH$_2$-MSVLTPLLRL GLTGSARRLP VPRAKCCOOH),\textsuperscript{39} it successfully detected the dynamic change in mitochondrial $O_2^-$ stimulated by various xenobiotics like phorbol 12-myristate 13-acetate (PMA),\textsuperscript{30,31,36} 2-methoxyestradiol (2-ME),\textsuperscript{40,41} and single-walled carbon nanotubes (SWCNTs).

The reaction scheme is shown in Figure 1a. In brief, fluorescein isothiocyanate (FITC) reacted with 2,4-dinitrobenzenesulfonyl chloride in dichloromethane (DCM) under the catalysis of trimethylamine. The obtained product was characterized by H$^\text{1}$ NMR (Figure S1a, ESI†) and MS (Figure S1b, ESI†) to confirm the high purity of the bi-substituted compound. This is important because the mono-substituted product is prone to hydrolysis and is reactive to thiol-containing compounds, both able to turn on its fluorescence.\textsuperscript{36,37} The bi-substituted compound, probe 1, absorbed poorly in between 450 to 520 nm (Figure 1b), and exhibited negligible fluorescence at $\lambda_{em}$ of 520 nm when excited at 494 nm (Figure 1c). After reaction with KO$_2$, the superoxide generator, the absorption and fluorescence emission spectra were similar to those from FITC at the same concentration (Fig. 1b & c). The probe reacts with O$_2^-$ very rapidly, reaching the plateau in less than 10 minutes after being mixed with KO$_2$ (Figure S2a, ESI†). The resulted fluorescence linearly increased with KO$_2$ concentration. As low as 0.65 μM KO$_2$ could be detected using 10 μM probe 1 (Figure S2b, ESI†). The signal levelled off once all the probes were consumed with KO$_2$ higher than 25 μM (Fig. S2a, ESI†). This result suggests that, two O$_2^-$ were needed to remove the two dinitrobenzenesulfonyl groups and convert one probe 1 molecule back to FITC.

Probe 1 has no targeting capability for mitochondria (MT). Therefore, we conjugated it to one of the MLS targeting peptide at the N-terminal via the reaction between –SCN and –NH$_2$. This peptide is the N-terminal portion of one of the endogenous nuclear-encoded MT proteins.\textsuperscript{39} This portion is responsible for transportation of such proteins into MT, via its interaction with the translocator inner membrane/translocator outer membrane protein complex. The selected peptide contains one lysine residue at the C-terminal that could also be labelled. HPLC analysis showed all free probe 1 had been conjugated with the peptide (Figure S3, ESI†).

The MLS-1 conjugate displayed comparable fold change to probe 1 in fluorescence intensity upon reaction with KO$_2$ (Figure 2a). The fluorescent product of probe 1 is fluorescein, the fluorescence of which is very sensitive to pH.\textsuperscript{43} We observed only background fluorescence from probe 1 or MLS-1 after reaction KO$_2$ if the solution pH was at 5.8. The fluorescence increased dramatically at pH 6.5, reaching the maximum at 8 and being stable up to pH 10 (Figure S4, ESI†). This result points out that our probe is suitable for superoxide measurement in cytosol (pH 7.0-7.5), endoplasmic reticulum (pH ~7.2), Golgi apparatus (pH ~ 6.6), and most importantly mitochondria (7.9-8.0).\textsuperscript{44,45} But it cannot be used to image superoxide in the acidic organelles like endosome and lysosome. The pH sensitivity of probe 1 also highlights the necessity of using the MLS peptide to deliver the probe to the right intracellular location. The MLS-1 conjugate maintained good selectivity to superoxide radicals over other common interferences like H$_2$O$_2$, OH, ONOO-, ClO-, and the iron ions (Figure 2b). Both probe-1 and MLS-1 showed no reactivity to GSH, a potential interfering compound present at substantial levels in cells.\textsuperscript{36,42}

We tested the cellular uptake of MLS-1 and its capability to detect O$_2^-$ change in cells. The Raw 264.7 macrophages (grown in a 24-well microtiter plate at an approximate density of 1×10$^5$/well) were treated sequentially with 20 μg/mL 2-ME for 4 hrs, 5 μM probe 1 or MLS-1 for 40min, and 5 μg/mL PMA for 30 min. The culture medium was refreshed after incubation with the probes. Thereafter, the cells were washed with 1×PBS for 3 times to remove any residual chemicals not entering the cells, and then incubated with CellLytic M, the mammalian cell lysis/extraction reagent from Sigma, for 15 min on a shaker. Upon cell lysis, an aliquot 60 μl of 50 mM phosphate buffer at pH 7.4 was added to each well to suspend the released probe 1 or MLS-1 before fluorescence measurement in Victor 2 Microplate Reader (Perkin Elmer). The results were shown on the left panel labelled as “without KO$_2$” in Figure 3. The control sample was not treated with either 2-ME or PMA, showing the base level of O$_2^-$ in cells. Some of the O$_2^-$ may have been generated during cell lysis. Nevertheless, the MLS-1 detected a significantly higher level of O$_2^-$ in the cells treated by 2-ME compared to the.
control cells. This chemical is an inhibitor of superoxide dismutase (SOD), the dominant working machine in converting superoxide into H₂O₂. Deactivating SOD caused accumulation of O₂⁻ in cells and thus a higher fluorescence signal was observed from MLS-1. The paired t-test confirmed that the fluorescence signals from MLS-1 in control cells and in cells treated with 2-ME were significantly different at the confidence level of 95% with a p value smaller than 0.005 (n = 3). Similarly, the O₂⁻ content was significantly higher in cells treated with PMA, a chemical known to stimulate superoxide generation, with a p value smaller than 0.01. The combined effect of 2-ME and PMA generated the highest level of O₂⁻ in cells.

Using probe 1, the overall fluorescence was very low, although noticeable increase in fluorescence was found in cells treated with PMA or with PMA+2-ME. The weak signal was due to its low concentration is sufficient to convert all probe molecules to the fluorophore structure. About 8 times more of the MLS-conjugate probe remained intense green fluorescence from MLS-1 in RAW 264.7 Macrophages. The fluorescence of images were recorded with the green (λ<sub>em</sub> = 500-535 nm) (a, d, g, j) and red (λ<sub>em</sub> = 594 nm; λ<sub>ex</sub> = 605-700 nm (b, e, h, k) channels, in unstimulated cells (a-c), and in cells stimulated by 2-ME (d-f) alone, PMA (g-i) or in combination with 2-ME (h-i). Overlaid images from the green and red channel shown in c, f, and i. Scale bar = 20 μm.

**Figure 4.** Confocal fluorescence images of MLS-1 co-stained with Mitotracker red CMXRos in RAW 264.7 Macrophages. The fluorescence of images were recorded with the green (λ<sub>em</sub> = 488 nm; λ<sub>ex</sub> = 500-535 nm) (a, d, g, j) and red (λ<sub>em</sub> = 594 nm; λ<sub>ex</sub> = 605-700 nm (b, e, h, k) channels, in unstimulated cells (a-c), and in cells stimulated by 2-ME (d-f) alone, PMA (g-i) or in combination with 2-ME (h-i). Overlaid images from the green and red channel shown in c, f, and i. Scale bar = 20 μm.

**Figure 5.** Confocal fluorescence images of MLS-1 co-stained with PO1 in RAW 264.7 Macrophages. The fluorescence of images were recorded with the green (λ<sub>em</sub> = 488 nm; λ<sub>ex</sub> = 500-535 nm) (a, c) and red (λ<sub>em</sub> = 543 nm; λ<sub>ex</sub> = 555-700 nm (b and d) channels were collected in cells stimulated by PMA (a-b) alone or in combination with 2-ME (c-d). Scale bar = 20 μm.

**Figure 6.** Confocal fluorescence images of MLS-1 in RAW 264.7 Macrophages stimulated by SWCNTs for 6 (a-b) or 12 hrs (c-d), acquired in the green (λ<sub>em</sub> = 488 nm; λ<sub>ex</sub> = 500-535 nm) Scale bar = 20 μm. It is also important that the intracellular sensor does not generate harmful effect to cells. Thus, we treated the Raw 264.7 macrophages at various probe 1 and MLS-1 concentrations (1, 2.5, 5, and 10 μM), and tested cell viability using the MTT assay. After 6, 12, or 24 hrs incubation with MLS-1, the viability of the Raw 264.7 cells was not affected (Figure S6, ESI†). Probe 1 at 10 μM reduced the cell viability to around 90% after 24 hrs incubation. While the superior safety of MLS-1 permits its usage as an intracellular sensor for O₂⁻, the MLS peptide should locate the probe right at MT, so that localized monitoring of radical production can be achieved. Dye localization was confirmed by co-staining the cells with both the superoxide sensor and Mitotracker<sup>TM</sup> Red (Invitrogen). Since probe 1 or MLS-1 was not fluorescent before reacting with superoxide, the cells were also treated by 2-ME and PMA to stimulate radical production so that MT localization could be observed clearly. The procedure was similar to the cell uptake experiment, except that the dye was mixed with Mitotracker Red before added to the cells. After all treatments, the cells were washed and subsequently fixed with 4% formaldehyde at 37 °C for 15 min before fluorescence observation by Leica S5P Inverted Confocal Microscope (Leica Microsystems, Inc.). Figure 4 showed the fluorescence images from MLS-1 (green) and Mitotracker (red), respectively. Both the green and red fluorescence were found in the cytosol around the nucleus, which was completely dark without being stained; and the overlaid images confirmed the co-localization of both MLS-1 and Mitotracker Red. On contrary, probe 1 was found at other places without red fluorescence, and even within the nucleus (Figure S7, ESI†). Agreeing with the cell lysis experiments, the confocal images clearly showed the enhanced green fluorescence while both PMA and 2-ME were used to stimulate the cells, compared to the control cells and to those treated only with 2-ME, under the same light acquisition settings.

Superoxide radicals have short life time because they will be converted to other ROS/RNS by enzymes. One of the main conversion routes is by SOD and the process forms H₂O₂. To demonstrate that our probe is suitable to image the dynamic change of superoxide radicals, we imaged this conversion process by using both our probe and the red H₂O₂ sensor from Tocris<sup>®</sup> Bioscience, peroxo orange 1 (PO1).<sup>30</sup> PO1 can be excited at 543 nm, and emits between 555 and 700 nm. Thus, we can image the fluorescence resulted from both O₂⁻ and H₂O₂ simultaneously using PO1 and MLS-1. Both dyes were lit up when the cells were incubated with PMA (Figure 5; fluorescence from PO1 was represented in red). However, in the cells treated by both PMA and 2-ME, the fluorescence from PO1 was much weaker, because 2-ME impeded with the conversion of O₂⁻ to H₂O₂, while the green fluorescence from MLS-1 remained intense and was accumulated at specific sites where the MT located. This result also serves as a convincing support for our probe’s high selectivity for superoxide radicals over H₂O₂.

Using this superoxide-specific and MT-targeting conjugate, MLS-1, we studied the oxidative stress induced...
by invasion of single-walled carbon nanotubes (SWCNTs). MT assay showed that incubating the RAW 264.7 macrophages with the carboxylated SWCNTs at concentration of 5, 10, 25, and 50 μg/mL caused significant cell death (Figure S6, ESIF): lower than 70% viability was observed with the highest SWCNT concentration during 6 hrs incubation, and also with the 3 higher SWCNT concentrations after 12 or 24 hrs incubation. Fluorescence imaging using MLS-1 for incubation with 10 and 25 μg/mL SWCNTs for 6 and 12 hrs were shown in Figure 6 (the images for other concentrations and incubation durations shown in Figure S8, ESIF). The green fluorescence from MLS-1 in MT increased gradually with the increase of SWCNT concentration and/or incubation duration. This result points out that SWCNT invasion enhanced superoxide production, which was rapidly converted to H2O2 (confirmed by PO1 staining; data not shown). Finally the elevated oxidative stress resulted in cell death.

The MLS-1 was also applied to image superoxide production in epithelial cells (Hela ATCC® CCL-2TM) (Figure S9, ESI†). Similar capability were observed, and the changes in superoxide levels caused by chemicals were clearly detected, demonstrating its applicability to diverse cell types.

Conclusions

We have generated a fluorescence probe that has excellent specificity at detecting superoxide radicals, and can specifically target MT without producing harmful effects. It can be used to monitor the dynamic change of superoxide radical levels in cells, as demonstrated in the present study using both small chemicals and carbon nanomaterials. The amine reactivity of probe 1 also permits labelling with other biomolecules such as antibodies or molecules targeting other organelles to detect generation of superoxide radicals at other locations inside the cells. Considering the central role of superoxide radical in cellular production of ROS/RNS, our probe can be a useful tool in deciphering its functions in cell signalling and host defense. Coupling with dyes specific for other ROS/RNS, it can also help with screening for chemicals that could damage enzymes involving in the conversion processes.

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Notes and references

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† Electronic Supplementary Information (ESI) available: experimental procedures, NMR and MS results, reaction rate investigation, detection calibration curve for probe 1 only; HPLC result to demonstrate purity of MLS-1, comparison of response with excess O2; * between 1 and MLS-1 in solution, cell viability when incubated with probe 1, MLS-1, and SWCNTs, imaging results for probe 1 intracellular location, images from MLS-1 to test SWCNT-induced generation of superoxide, and all results obtained in HeLa cells. See DOI: 10.1039/c000000x/