Fluorescent Probes for H_2O_2

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Lighting up H_2O_2: The Molecule that Is a "Necessary Evil" in the Cell

*Wei Zhao**

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2008 was an exciting year for scientists in the fluorescence imaging community, who witnessed the award of the Noble Prize in Chemistry to three scientists for the discovery and development of a fluorescent protein for living-cell visualization. A recent report on a new fluorescent probe to image mitochondrial hydrogen peroxide (H_2O_2) in living cells by Chang et al.[1] has brought more excitement to the community.

H_2O_2 has been called a "necessary evil"[2] because of its dual character of adverse and, more recently discovered, beneficial activities.[1–5] As a major reactive oxygen species (ROS) in living organisms, its overproduction in cellular mitochondria is implicated in the development of many severe diseases such as cancer and neurodegenerative Parkinson's and Alzheimer's diseases.[1,3] A more recent study suggests that H_2O_2 is responsible for the anticancer activity of vitamin C.[5] As a second messenger, H_2O_2 determines lifespan[4] and plays an important role in intracellular signaling linked to redox-based mechanisms.[2,4] As the "signaling face" of ROS, H_2O_2 shows its specificity in biological activity as suggested by its dual mediator function in both cell growth and apoptosis. As pointed out by Giorgio et al.,[4] the specificity can be determined through three pathways by investigating 1) the intensity of the pro-oxidant challenge, 2) the intracellular site of production, and 3) the local variations in H_2O_2 concentration that could be crucial for the activation of specific targets. However, the authors thought that identifying the locations and quantifying H_2O_2 fluxes in living cells was virtually impossible with the technologies available at that time.[4] Now, with the breakthrough in creating new multifunctional fluorescent probes by Chang et al.,[1] unraveling the mysteries of mitochondrial H_2O_2 specificity is within reach.

Since the discovery of H_2O_2 in 1818, fluorescent molecular probes for H_2O_2 have been developed in numerous studies; these range from the traditional dihydro compounds, such as dihydrorhodamine and 2′,7′-dichlorodihydrofluorescein,[1,6] to the most recent nanostructural materials, such as carbon nanotubes.[7] The traditional fluorescent probes have been used widely for the fluorometric detection of H_2O_2, but they are not specific for H_2O_2 and tend to react with various other ROS to give a fluorescence response. Recently, a few new fluorescent probes capable of selectively detecting H_2O_2 have been synthesized. These include pentafluorobenzenesulfonyl fluoresceins, Peroxyfluor-1, aminocoumarin masked by the butanediol ester of a *p*-dihydroxyborylbenzyloxycarbonyl derivative, 7-hydroxy-2-oxo-*N*-(2-(diphenylphosphino)ethyl)-2*H*-chromene-3-carboxamide, and a Eu^{3+} tetracycline complex.[6] Some of these probes have been used to monitor intracellular H_2O_2 levels.[6]

The fluorescent probes listed above are specific to H_2O_2; however, none of them selectively targets cellular mitochondria. In recent years, Chang's group has synthesized a family of boronate-based probes, red-fluorescent Peroxyresorufin-1, green-fluorescent Peroxyfluor-1, and blue-fluorescent Peroxyxanthone-1.[6] The selectivity of these probes to H_2O_2 is based on the conversion of an arylboronate group into a phenol group (Figure 1), selectively mediated by H_2O_2. These boronate-based fluorescent probes have been evaluated recently as the only contrast agents for detecting hydrogen peroxide at physiological concentrations with high specificity.[8] However, it was also pointed out that their potential for in vivo imaging is limited owing to their low tissue penetration.[8] As summarized in the Invitrogen Fluorescent Molecular Probe Handbook,[9] there are nine commercial H_2O_2 fluorescent molecular probes and thirty mitochondrial fluorescent probes. However, none has been developed for specifically targeting mitochondrial H_2O_2.

To fill this gap, Chang's group has developed the fluorescent probe Mitochondria peroxy yellow 1 (MitoPY1) for selectively imaging mitochondrial H_2O_2 in live cells.[1] The group has created a bifunctional dye containing both a mitochondria-targeting lipophilic cation (red in Figure 1), the triphenylphosphonium head group, and a peroxide-responsive element (green in Figure 1), the boronate moiety. The introduction of lipophilic phosphonium cations resolves the membrane-penetration issue. The cations can easily move through phospholipid bilayers because of the low activation energy for passing through the hydrophobic barrier of the mitochondrial inner membrane; ionophores are not needed and the probe accumulates in the mitochondria, driven by the membrane potential.[10] At the same time the chemispecific boronate group can selectively respond to H_2O_2 over other

[*] Prof. W. Zhao
Department of Chemistry, University of Arkansas at Little Rock, 2801 South University Avenue, Little Rock, AR 72204 (USA)
Fax: (+1) 501-569-8838
E-mail: wxzhao@ualr.edu
Homepage: http://www.ualr.edu/wxzhao

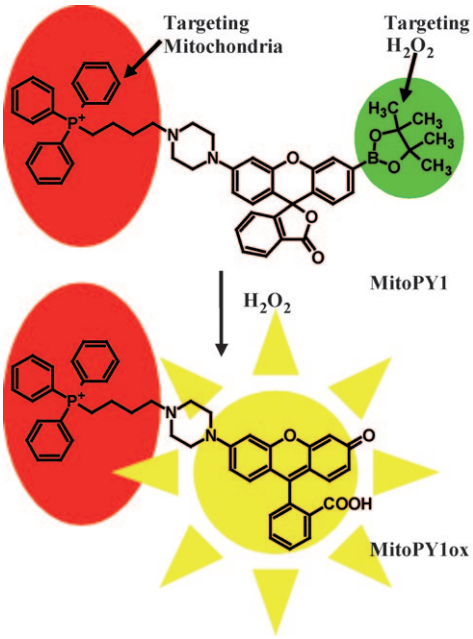

Figure 1. Design of the multifunctional fluorescent probe MitoPY1 which has a mitochondria-targeting phosphonium group and a peroxide-responsive boronate switch. Reaction of MitoPY1 selectively with mitochondrial H_2O_2 triggers an increase in fluorescence by the conversion of MitoPY1 to MitoPY1ox.

ROS such as superoxide, hydroxyl radical, and nitric oxide.[1,6] Reaction of MitoPY1 selectively with mitochondrial H_2O_2 triggers an increase in fluorescence by the conversion of MitoPY1 to MitoPY1ox (Figure 1), which gives enhanced fluorescence at $\lambda = 528$ nm upon excitation at $\lambda = 510$ nm.

Chang et al. have tested the synthetic MitoPY1 probe in four types of mammalian cell lines including cervical cancer HeLa, Cos-7, HEK293, and CHO.K1 cell lines.[1] To make sure the probe targets the mitochondria, a commercial mitochondrial indicator, MitoTracker Deep Red 633 was used as a control. Additionally, a lysosomal indicator, LysoTracker Red, was also used to ensure that the new probe targets the mitochondria only. During the experiments, Chang et al. monitored the cells using both brightfield measurements and nuclear staining with Hoechst 33342 to ensure that the cells are viable throughout the experiments. To demonstrate that the selectivity for targeting mitochondrial H_2O_2 is a unique property of MitoPY1, they conducted control experiments with the product MitoPY1ox, which showed no enhanced fluorescence upon the addition of H_2O_2 in the living cells. To further demonstrate the specificity of MitoPY1 for targeting mitochondria, a control fluorescent probe lacking the mitochondria-targeting lipophilic cation, ContPY1 (Figure 2), was synthesized by replacing the phosphonium head group with an acetyl group. The probe showed fluorescence turn-on response to H_2O_2 in the living cells because of its boronate switch; however, no preferential targeting to cellular mitochondria was observed. The group further confirmed the selectivity of MitoPY1 for mitochondrial H_2O_2 in a larger population of living cells by running complementary flow cytometry experiments.

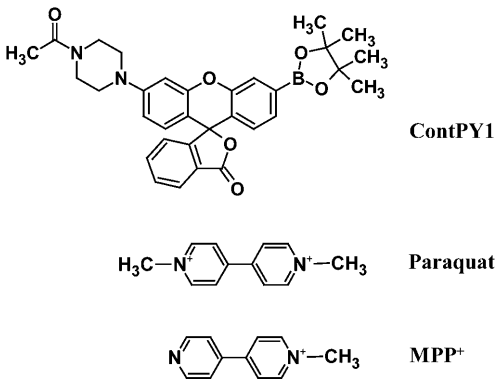

Figure 2. Chemical structures of ContPY1, paraquat, and MPP^+.

Finally, Chang et al.[1] applied this new probe to image the endogenous production of mitochondrial H_2O_2 in living HeLa cells, induced by the pesticide paraquat (1,1′-dimethyl-4,4′-bipyridinium, Figure 2). Paraquat is a potential neurotoxicant linked to Parkinson's disease, and its chemical structure closely resembles that of 1-methyl-4-phenylpyridinium ion (MPP^+; Figure 2). Because MPP^+ is the toxic metabolite that mediates the effects of the Parkinsonism-inducing agent 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), the chemical similarity between paraquat and MPP^+ points to paraquat as a potential environmental toxin involved in Parkinson's disease.[11] Chang et al. showed that MitoPY1 is sensitive enough to detect the increasing mitochondrial H_2O_2 level in living HeLa cells after exposure to paraquat at 1 mM, a concentration lower than paraquat's half maximal inhibitory concentration of 1.02 mM in HeLa cells. The results suggest that the MitoPY1 probe can be used to study oxidative stress induced by environmental toxins in H_2O_2-implicated diseases.

To address the biological specificity of H_2O_2,[4] the current work provides a means to identify the molecule's location and quantifying its flux in living cells through the design of fluorescent probes with selected functional groups that may specifically and precisely target subcellular locations. As the "signaling face" of biologically relevant ROS, H_2O_2 in the O_2 reduction pathway has the lowest reactivity with reduction potential of 0.32 V, the highest stability with a half-life of 10^{-5} s, and the highest intracellular concentration which may span four orders of magnitude from 10^{-8} M in proliferation to 10^{-4} M in apoptosis.[4] There is urgent demand for a sensitive and specific probe for H_2O_2 that allows not only quantitative but also dynamic assessment of this signaling molecule in live cells.[2] The molecular probes currently available may provide unique features including photostability, selectivity, specificity, and stability under physiological conditions. With the encouragement of the current breakthrough, new probes possessing additional characteristics of fast response, large dynamic concentration range, and reversibility may be created to achieve the ultimate goal, unraveling the mysteries of mitochondrial H_2O_2.

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[1] B. C. Dickinson, C. J. Chang, *J. Am. Chem. Soc.* **2008**, *130*, 9638 – 9639.
[2] S. G. Rhee, *Science* **2006**, *312*, 1882 – 1883.
[3] M. T. Lin, M. F. Beal, *Nature* **2006**, *443*, 787 – 795.
[4] M. Giorgio, M. Trinei, E. Migliaccio, P. G. Pelicci, *Nat. Rev. Mol. Cell Biol.* **2007**, *8*, 722 – 728.
[5] Q. Chen, M. G. Espey, A. Y. Sun, C. Pooput, K. L. Kirk, M. C. Krishna, D. B. Khosh, J. Drisko, M. Levine, *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 11105 – 11109.
[6] N. Soh, *Anal. Bioanal. Chem.* **2006**, *386*, 532 – 543.
[7] Y. Xu, P. E. Pehrsson, L. Chen, W. Zhao, *J. Am. Chem. Soc.* **2008**, *130*, 10054 – 10055.
[8] D. Lee, S. Khaja, J. C. Velasquez-Castano, M. Dasari, C. Sun, J. Petros, W. R. Taylor, N. Murthy, *Nat. Mater.* **2007**, *6*, 765 – 769.
[9] The web edition of the handbook: A Guide to Fluorescent Probes and Labeling Technologies, 10th Edition, Invitrogen, http://probes.invitrogen.com/handbook.
[10] M. P. Murphy, R. A. J. Smith, *Annu. Rev. Pharmacol. Toxicol.* **2007**, *47*, 629 – 656.
[11] A. L. McCormack, M. Thiruchelvam, A. B. Manning-Bog, C. Thiffault, J. W. Langston, D. A. Cory-Slechta, D. A. Di Monte, *Neurobiol. Dis.* **2002**, *10*, 119 – 127.

