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Highly Selective Excited State Intramolecular Proton Transfer (ESIPT)-Based Superoxide Probing

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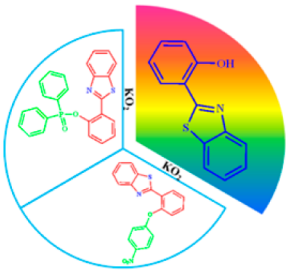
ABSTRACT



Two novel fluorescent probe conjugates of 2-(benzothiazol-2-yl)-phenol (HBT) enable ratiometric and selective superoxide detection by an established *excited state intramolecular proton transfer* (ESIPT) mechanism giving a 60-fold intensity increase.

Reactive oxygen species (ROS) play a decisive role in neurodegenerative disorders (e.g., Alzheimer's disease and Parkinson's disease) and also widely in biological systems and in nature. Because of these significant issues related to health and the environment, selective and sensitive detection of these species has drawn much recent research attention.[1] Along with the neurodegenerative diseases, reactive oxygen species (ROS)[2] are also a key factor in other pathologies including cancer, diabetes, and simply aging.[3] Reactive oxygen species (ROS) include superoxide, hypochlorite, hydrogen peroxide, hydroxyl radical, nitric oxide, and peroxynitrite. Superoxide ($O_2^{\bullet-}$) is a fascinating analyte in that it is both a radical and anion. It has a short half-life and can combine easily with NO· to give peroxynitrite. Real-time detection of superoxide is vital in revealing the origin of a range of physiological processes in living organisms, such as aging, muscle fatigue, ischemia-reperfusion, and inflammation.[4,5] The detection of superoxide with high selectivity and sensitivity is an ongoing challenge. Different kinds of fluorescent molecular probes have been synthesized previously; most of them reported to date are BODIPY-, fluorescein-, and cyanine-based species involving cleavable groups.[6]

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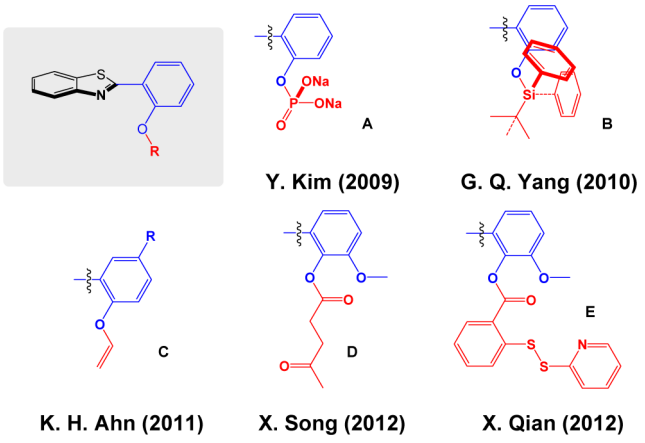

Figure 1. Previously known HBT probes based on ESIPT.[7]

In this report, we present two novel flurescent probes for superoxide detection based on phosphinate and ether hydrolysis. Recent reports have involved chemodosimeters based on superoxide-induced oxidation reactions,[8a−c] reactions with nitroxide,[8d] and deprotection of the 2,4-dinitrobenzenesulfonyl[8e] and phosphinate groups.[9,10] As per our knowledge, this is the first example of (*i*) a superoxide sensor based on *excited state intramolecular proton transfer* (ESIPT),[11] and (ii) one involving the reaction of superoxide with an ethereal group. Some of the reported probes based on ESIPT are shown in Figure 1. Superoxide has been coined a *super nucleophile*[12] which permits hydrolysis of phophinates and the 4-nitro ether by way of nucleophilic addition reactions. Also, fluorescein-based phophinates have been used as probes for the detection of superoxide by this same principle.[9,10] Herein, we have chosen a probing modality based on a simple deprotection phenomenon: the hydroxyl group of 2-(benzothiazol-2-yl)-phenol (HBT)[13] (Figure 2) in which the phosphinate group [$P(O)Ph_2$] and 4-nitro ether involves the detachment via hydrolytic action by superoxide through an addition−elimination reaction (Figure 3) giving free 2-(benzothiazol-2-yl)-phenol (HBT) as a final fluorescent product. 2-(Benzothiazol-2-yl)-phenol (HBT) is very well-known as an intramolecularly hydrogen bonded molecule exhibiting *excited state intramolecular proton transfer* (ESIPT)[11] in which rapid photoinduced proton transfer results in tautomerization. The tautomerization depicted in Figure 3 affords a large bathochromic shift. Also it resembles THT (Thioflavin-T), a well-known dye to aid in visualizing plaques formed by misfolding of the amyloid protein.

The probes were obtained by commercially available diphenylphosphinic chloride ($Ph_2P(O)Cl$) and 1-chloro-4-nitrobenzene with HBT, in one convenient step under facile reaction conditions; probes **1** and **2** were obtained in excellent purity and good yield (68% and 54%). These probes were characterized by ^1H and ^{13}C NMR spectroscopy and mass spectrometry (Supporting Information). 2-D NMR spectroscopic data were also obtained to allow for more definitive atomic assignments on the solution structure of probe **1** (Supporting Information).

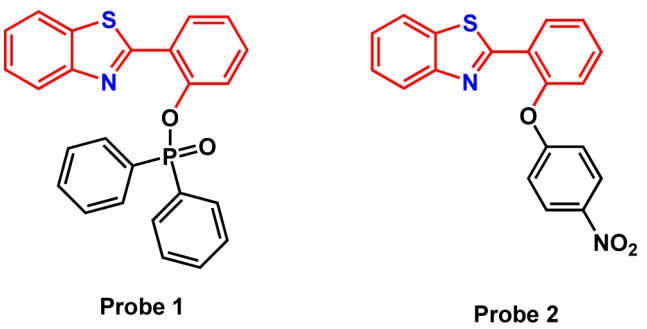

Figure 2. Structures of probes **1** and **2**.

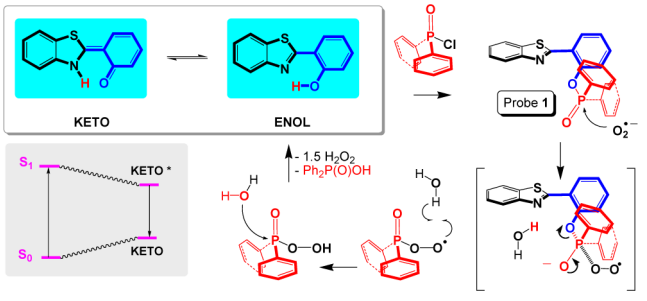

Figure 3. Synthesis of probe **1** from 2-(benzothiazol-2-yl)-phenol (HBT), its deprotonation, and a depiction of the ESIPT mechanism involving plausible ROS-based byproducts.

Spectroscopic properties of probes **1** and **2** were obtained under physiological conditions. First, the probe was dissolved in DMSO[14] and subsequently diluted in 50%

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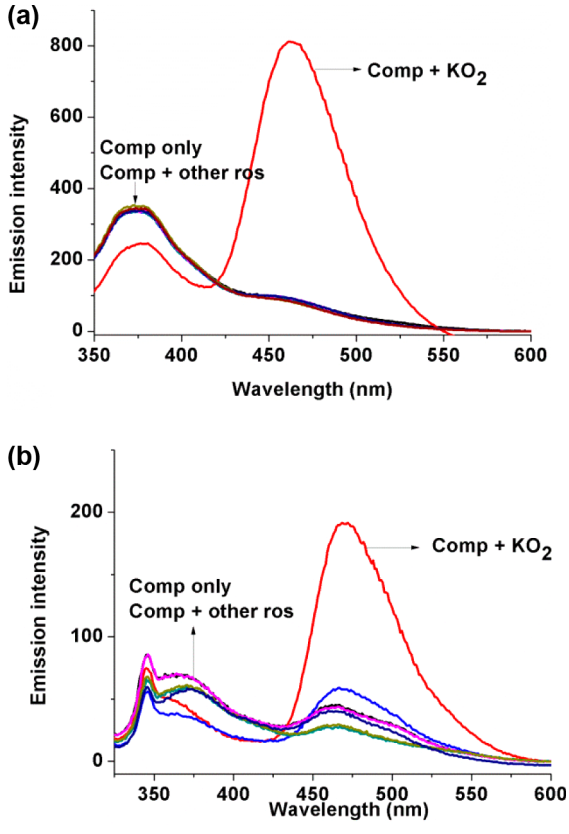

Figure 4. Emission spectra of (a) Probe **1** (5×10^{-6} M, buffered H_2O/DMSO 50:50; pH 7.2; 10 mM HEPES buffer) and (b) probe **2** (5×10^{-6} M, H_2O/DMSO 50:50) with (ROS) KO_2, H_2O_2 (30% in water), NaOCl (30% in water), ^{t}BuOOH (5.0−6.0 M in decane), •OH (10 mg of $FeSO_4$ in 0.1 M H_2O_2 solution), •O^{t}Bu (10 mg of $FeSO_4$ in 0.1 M ^{t}BuOOH solution), *m*-CPBA (~10 equiv) incubated for 10 min at rt. λ_{exci} = 310 nm, slit width Ex, Em = 5.

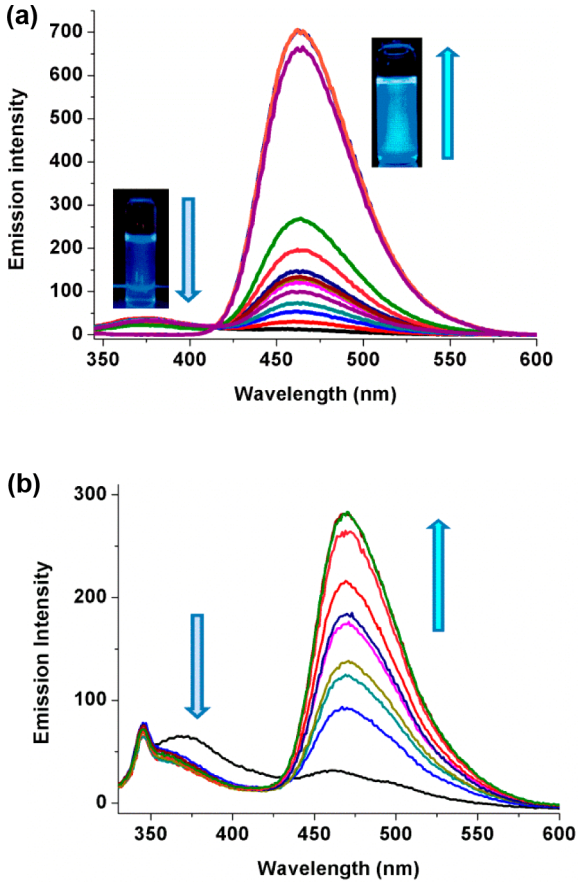

Figure 5. Emission spectra of (a) probe **1** (5.0×10^{-6} M, buffered H_2O/DMSO 50:50; pH 7.2; 10 mM HEPES buffer) and (b) probe **2** (5×10^{-6} M, H_2O/DMSO 50:50) with increasing KO_2 analyte incubated for 10 min at rt. λ_{exci} = 310 nm; slit width Ex, Em = 1.5.

HEPES buffer (10 μM, pH.7.2). Analyte detection properties of probes were assessed by UV−vis absorption and emission spectroscopy (Supporting Information). The presence of the phosphinate and 4-nitro ether groups allowed us to explore a secondary ROS screening in which deprotection gives back the parent 2-(benzothiazol-2-yl)-phenol (HBT). The fluorescence quantum yield of HBT in DMSO was found to be ($\Phi = 0.083 \pm 0.005$) (Fluorescein in 0.1 M was used as a standard). Probes **1** and **2** were tested with reactive oxygen species (ROS) *m*-CPBA, KO_2, H_2O_2, NaOCl, ^{t}BuOOH, •OH, and •O^{t}Bu. With the addition of small portions of superoxide only, there was a dramatic increase in fluorescence intensity involving an ~58-fold increase and a bathochromic shift of 85 nm relative to probe **1** (Figure 4a). A 6-fold increase and bathochromic shift of 101 nm was found relative to probe **2** (Figure 4b). Superoxide gave a considerable change in emission intensity through phosphinate and 4-nitro ether deprotection. In this regard, a reaction of the probe with 1 equiv of KO_2 was performed. After filtration of the reaction mixture, the filtrate was then subjected to a ^{31}P NMR spectroscopic study in which a singlet (δ 33.40) assigned to the phosphinate phosphorus completely disappears (Supporting Information), indicating full phosphinate deprotection (Figure S4). When tested with the increasing concentration of superoxide there was a steady increase in fluorescence intensity with the increase in concentration of superoxide (Figure 5). By using these data we have calculated the detection limit, which is found to be 3.0×10^{-3} M and 6.38×10^{-5} M for probe **1** and **2**, respectively.

To obtain biological insight with these probe systems, the cytotoxicity of probe **1** was examined with SH-SY5Y cells. The neuroblastoma cells were treated with probe **1** in the range 1−36 μM in media for 1.0 h. Compared to control values (CTL or Alc CTL), there was no significant decrease in cell viability (%) induced by probe **1** in this media. Cell viability was measured using an MTT solution (MTT = 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 1.0 mg/mL) which was added to each vial

(14) DMSO is used as solvent because the probe was sparingly soluble in water only. Importantly, the nucleophilic reaction rate is solvent dependent. Polar aprotic solvents like DMSO are better for this reaction: these solvents will not form strong hydrogen bonding networks with the nucleophile, allowing for facile nucleophilic attack.

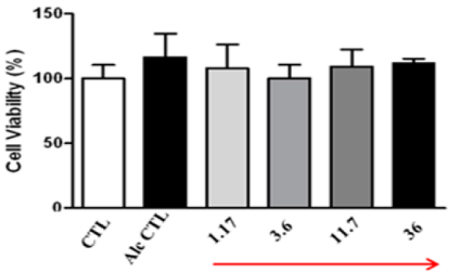

Figure 6. Cell viability (%) of SH-SY5Y cells in response to probe **1** determined by MTT assaying. SH-SY5Y cells were exposed to probe **1** (1−36 μM) for 1 h; cell viabilities were compared to control values (CTL = 100%). Data are expressed as means ± STD of three independent measurements. CTL = control; Alc CTL = alcohol control.

and then incubated with cells at 37 °C for 4.0 h. Formazan crystals were first dissolved in dimethyl sulfoxide. UV−vis absorbances were then measured at 570 nm using a microplate reader (Tecan, Maennedorf, Switzerland). This assay supports the utility of the probe for use in biological systems in detecting superoxide without short-term damage to the neuronal systems (Figure 6).

In conclusion, two novel fluorogenic conjugates of 2-(benzothiazol-2-yl)-phenol (HBT) were synthesized in one facile step; probes **1** and **2** act as ratiometric, fluorescent, and selective molecular recognition devices for the detection of superoxide in combined aqueous media. There is a bathochromic shift of 85 nm and nearly a 60-fold increase in fluorescence intensity. These probes are highly selective for the detection of superoxide without interference of any other ROS; the MTT assay for cell viability of probe **1** demonstrated probe non-neurotoxicity as tested using SH-SY5Y cells. Also, this is a novel superoxide sensing approach by *ether* hydrolysis giving a detection limit of 6.38×10^{-5} M.

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Supporting Information Available. Methods, experimental procedures, additional spectral data. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.