LETTER

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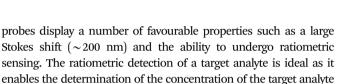
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A simple ESIPT-based fluorescence probe (HMBT-LW) was developed for the detection of superoxide (O_2^{*-}). HMBT-LW was synthesised over two steps and was shown to rapidly detect low concentrations of O_2^{*-} (limit of detection = 7.4 μ M), fully reacting within two minutes. Furthermore, HMBT-LW demonstrated excellent selectivity and sensitivity towards O_2^{*-} .

Reactive oxygen species (ROS) are the transient by-products generated from the electron transport chain.¹ More specifically, the ROS, superoxide $(O_2^{\bullet-})$ is a anion radical generated from the single electron reduction of molecular oxygen (O_2) , which means $O_2^{\bullet-}$ is the precursor to most ROS.² $O_2^{\bullet-}$ is capable of reacting with nitric oxide (NO[•]), which generates the highly reactive nitrogen species peroxynitrite (ONOO⁻), or with superoxide dismutase (SOD) to produce hydrogen peroxide (H_2O_2) . Hydrogen peroxide can then be transformed into the highly reactive hydroxyl radical (*OH) and hypochlorous acid (HOCl). These reactive oxygen species are associated with a number of pathological processes, including cardiomyopathy, autism, diabetes mellitus, cancer and neurodegenerative disorders (e.g., Alzheimer's disease and Parkinson's disease).³⁻⁶ Therefore, the development of a fluorescence probe for the real-time detection of $O_2^{\bullet-}$ would further aid the understanding of $O_2^{\bullet-}$ related diseases in living organisms.

Excited-state intramolecular proton transfer (ESIPT) is widely used in the design of fluorescent probes⁷ as ESIPT-based fluorescent

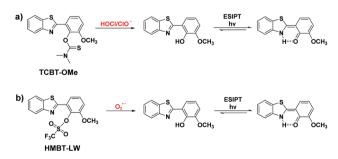
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directly without need of calibration.⁷⁻⁹ Within our research group, we have developed several ESIPT-based fluorescent probes for the detection of biological reactive oxygen species as well as biological thiols.^{10–13} Previously, we have developed a thiocarbamate functionalised methoxyhydroxybenzothiazole (**HMBT**) fluorescent probe **TCBT-OMe** for the detection of HOCl/ClO⁻ (Scheme 1). The addition of HOCl/ ClO⁻ to **TCBT-OMe** resulted in the rapid hydrolysis (<10 s) of the thiocarbamate linker, leading to a ratiometric change in fluorescence intensity.¹²

Most fluorescent probes that are reported for the detection of $O_2^{\bullet^-}$ utilise its nucleophilicity to achieve excellent selectivity over other ROS.^{14–20} As a result of this, we believed the functionalisation of **HMBT** with the $O_2^{\bullet^-}$ reactive trifluoromethanesulfonate unit would result in a ratiometric fluorescent probe for the detection of $O_2^{\bullet^-}$ (Scheme 1).²¹

HMBT-LW was synthesized over two steps. The first step of the synthesis involved the addition of a 2:1 aq H_2O_2/aq HCl solution to 2-aminothiophenol and *o*-vanilin in EtOH, which formed **HMBT** in good yield (68%).^{22–24} With **HMBT** in hand,



Scheme 1 (a) Our previously reported ESIPT probe for the detection of HOCl/ClO⁻. (b) This work – a trifluoromethanesulfonate linker-based ESIPT **HMBT-LW** for the detection of $O_2^{\bullet-}$.



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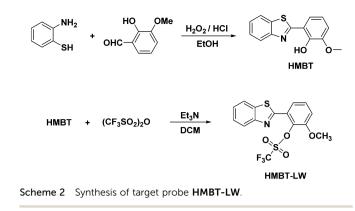
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trifluoromethanesulfonic anhydride was then added dropwise into a solution of **HMBT** in DCM at -78 °C under argon, NEt₃ was subsequently added to the reaction. This reaction proceeded smoothly furnishing **HMBT-LW** in good yield (52%) (Scheme 2). The chemical structure of **HMBT-LW** was fully characterized by ¹H NMR, ¹³C NMR and high-resolution mass spectrometry (HRMS).

Next, we evaluated the UV-Vis properties of HMBT-LW $(5 \,\mu\text{M})$ with the addition of $O_2^{\bullet-}$ (42 equivalents). This addition led to an increase in UV absorption between 200-400 nm indicating a change chemical structure (Fig. S1, ESI[†]). We then turned our attention towards the ability of HMBT-LW to detect O2^{•-} using fluorescence. Remarkably, HMBT-LW was shown to have a rapid response towards $O_2^{\bullet-}$ with a significant increase in fluorescence intensity being observed within 2 minutes (Fig. S5, ESI[†]). Initially, a fluorescence emission intensity at 378 nm was only observed, since the ESIPT process is blocked by the trifluoromethanesulfonate group. However, in the presence of $O_2^{\bullet-}$, a notable increase in fluorescence emission intensity at 483 nm and a simultaneously decrease in fluorescence emission intensity at 378 nm was observed (Fig. 1) corresponding to the deprotection and release of the HMBT fluorophore enabling the ESIPT process to take place (Reaction mechanism confirmed by HRMS - see Fig. S6 and S7, ESI[†]). In addition a quantum yield of 0.508 was determined for HMBT under these measurement conditions.25

HMBT-LW was then shown to have good stability over a range of different pH 4–10, (Fig. S3, ESI[†]) and was capable of detecting low concentrations of $O_2^{\bullet^-}$ with a Limit of Detection (LoD) of 7.4 μ M (Fig. S4, ESI[†]). Furthermore, **HMBT-LW** demonstrated excellent selectivity towards $O_2^{\bullet^-}$ over other ROS and biologically relevant analytes (Fig. 2).

With this research we have developed an ESIPT-based fluorescence probe (**HMBT-LW**) for the selective and sensitive detection of $O_2^{\bullet-}$. Sadly, the excitation wavelength for **HMBT-LW** is too short to enable its use in cellular imaging experiments. However, we are currently exploring related ESIPT based systems with longer excitation wavelengths that are more suitable for cellular imaging experiments. In summary **HMBT-LW** provides a platform on which it will be possible to develop long wavelength ESIPT-based fluorescent probes for the ratiometric selective and sensitive detection of $O_2^{\bullet-}$.

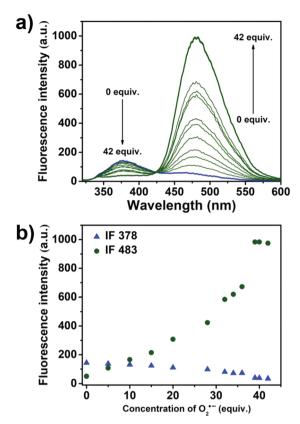


Fig. 1 (a) Changes in fluorescence emission intensity of **HMBT-LW** (5 μ M) with increasing additions of O₂^{•-} (from 0 to 42 equiv.) in PBS buffer solution (10 mM, V/V, DMSO/PBS = 1/1, pH = 7.4) after 3 min. (b) Emission at 378 and 483 nm of **HMBT-LW** (5 μ M) with increasing addition of O₂^{•-} (from 0 to 42 equiv.) in PBS buffer solution (10 mM, V/V, DMSO/PBS = 1/1, pH = 7.4) after 3 min. λ_{ex} = 310 nm. Slit widths: ex = 8 nm, em = 5 nm.

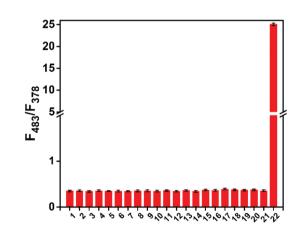


Fig. 2 Fluorescence intensity ratio changes (based on the peak heights at the maxima, 378 and 483 nm respectively) with addition of $O_2^{\bullet-}$ (39 equiv.) and other interfering reagents (120 μ M). 1. Probe only; 2. ClO⁻; 3. H₂O₂; 4. •OH; 5. ${}^{1}O_2$; 6. ONOO⁻; 7. ROO[•]; 8. H₂S; 9. glucose; 10. GSH; 11. Cys; 12. Hcy; 13. Na⁺; 14. K⁺; 15. Ca²⁺; 16. Mg²⁺; 17. Zn²⁺; 18. Fe²⁺; 19. Al³⁺; 20. Cu²⁺; 21. Fe³⁺; 22. O₂^{•-}. λ_{ex} = 310 nm. Error bar represents s.d. Slit widths: ex = 8 nm, em = 5 nm. 30 min wait between measurements.

Conflicts of interest

No conflicts of interest.

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