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ESIPT-based fluorescence probe for the rapid detection of peroxynitrite 'AND' biological thiols

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An ESIPT-based AND logic fluorescence probe (GSH-ABAH) was developed for the simultaneous detection of $ONOO^-$ and biological thiols. GSH-ABAH was shown to have good cell permeablility and with the addition of just SIN-1 ($ONOO^-$ donor) or GSH, no fluorescence response was observed in live cells. However, in the presence of both analytes GSH-ABAH could be used to image exogenous $ONOO^-$ and GSH added to RAW264.7 cells.

Peroxynitrite ($ONOO^-$) is a highly reactive nitrogen species[1] with an incredibly short biological half-life (< 10 ms).[2] $ONOO^-$ is known for its deleterious effects, causing irreversible damage to a range of biological targets such as lipids, proteins and nucleic acids.[3] As a result, abnormal concentrations of $ONOO^-$ are thought to be associated with inflammation, cancer, atherosclerosis and neurodegenerative diseases.[4-7] In addition, biological thiols such as glutathione (GSH) and cysteine (Cys) are essential in maintaining biological redox homeostasis.[8-10]

GSH is a natural tripeptide (γ-L-glutamyl-cysteinyl-glycine) that exists in the thiol reduced form (GSH) and disulphide-oxidised (GSSG) form.[11] GSH is the predominant form, which exists in mammalian and eukaryotic cells where it functions as an antioxidant.[12-14] More importantly, GSH serves as an $ONOO^-$ scavenger through its direct oxidation by $ONOO^-$.[15] Therefore, it is common to find elevated levels of GSH when cells are undergoing oxidative stress. Therefore, the susceptibility of a cell towards $ONOO^-$ largely depends on the concentration of intracellular GSH.[7, 16, 17]

Within our research groups, we are interested in developing small molecule fluorescent probes for the detection of biological reactive oxygen species as well as biological thiols.[18-21] While many literature reported fluorescent probes have been used to understand the roles of single chemical species, which include metal ions[22] and reactive oxygen species[23, 24] in biological systems.[25] Relatively, few probes have been developed to report on the role of two or more analytes in a biological system. In parallel to the development of fluorescent probes, the field of molecular logic gates has developed.[26, 27]

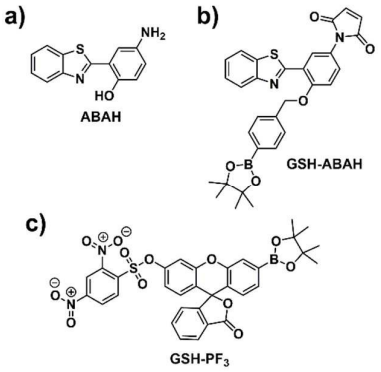

Fig. 1 (a) **ABAH** ESIPT fluorophore previously used in the literature (b) This work – ESIPT-based probe **GSH-ABAH** for the detection of $ONOO^-$ and biological thiols (c) Structure of the **GSH-PF3** probe previously used for the simultaneous detection of $ONOO^-$ and GSH

Molecular logic gates are molecules that have the ability to bind to multiple analytes and transform the multiple binding events to a measurable output. Recently, we have developed dual activated fluorescent probes. Where, the 'AND' logic operation requires two analytes to produce a positive output signal. These 'AND' logic systems have the ability to detect two different analytes within the same biological sample and hence provide a simple approach for monitoring complex bimolecular

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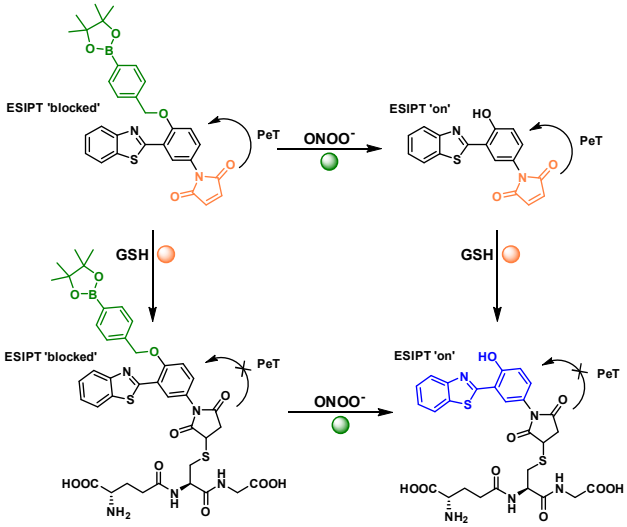
events, where two species may be intimately responsible for a particular disease.[28]

Dual fluorescence based probes for monitoring the relationship between $ONOO^-$ and GSH are uncommon,[29, 30] despite numerous fluorescence based probes being developed for the sensing of these analytes seperately.[31 32] Recently, we have developed a fluorescein-based 'AND' logic gate, which was capable of detecting $ONOO^-$ 'AND' GSH in cells (Fig 1c).[33] 'AND' logic based fluorescence probes for $ONOO^-$ 'AND' GSH are of particular interest as they could potentially be used to evaluate the therapeutic efficacy of a particular treatment towards Alzheimer's disease.[34]

In this work, we set out to improve on our earlier system by developing an intramolecular proton transfer (ESIPT) 'AND' logic gate for the simultaneous detection of $ONOO^-$ 'AND' GSH. Owing to the attractive characteristics of ESIPT fluorophores, which include: ratiometric sensing, large stokes shift and environmental sensitivity. Essentially, if a ratiometric system could be developed then this would be a significant advance, potentially allowing for calibration free monitoring.[35-37]



Scheme 1. Fluorescence turn 'on' mechanism of **GSH-ABAH** in the presence of $ONOO^-$ and GSH.

4-Amino-2-(benzo[d]thiazol-2-yl)phenol (**ABAH**) was regarded as an ideal ESIPT fluorophore for the development of an 'AND' based fluorescence probe due to having a free phenol and amino group, which can be independently derivatized (Fig. 1, Scheme S1).[36, 38-41] We believed the functionalization of the free phenolic unit of **ABAH** with a benzyl boronic ester would block the ESIPT process and serve as the reactive unit for $ONOO^-$. Due to aromatic boronates having a greater reactivity towards $ONOO^-$ over $HClO/ClO^-$ and H_2O_2.[42] Previously, the functionalization of the amino group of **ABAH** with the thiol-reactive maleimide group resulted in the quenching of the fluorescence intensity due to a PET process. However, in the presence of biological thiols the fluorescence intensity was rapidly restored.[43] Therefore, we thought that the combination of these two reactive units with **ABAH** would result in an effective PET+ESIPT 'AND'-logic probe for the detection of $ONOO^-$ AND biological thiols (Fig. 1, Scheme 1).

To test this hypothesis, we synthesized probe **GSH-ABAH** over three steps (Scheme S2 – see ESI). **ABAH** was first synthesized in excellent yield (73%) by heating 2-aminophenol and *p*-aminosalicylic acid in polyphosphoric acid (PPA) at 180 ^oC. With **ABAH** in hand, maleic anhydride was then added to a solution of **ABAH** in glacial acetic acid. This condensation reaction was performed under reflux for 4 hours to afford the desired intermediate **2** as a yellow solid. **2** was then alkylated using (4-Bromomethylphenyl)boronic acid pinacol ester and K_2CO_3 in DMF to afford **GSH-ABAH** in 27% yield (Scheme S2). The chemical structure of **GSH-ABAH** was fully characterized by ^1H NMR, ^{13}C NMR and high resolution mass spectrometry (HRMS).

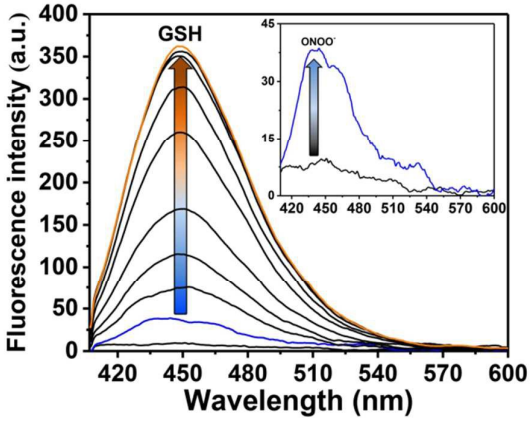


Fig. 2 – Fluorescence spectra of **GSH-ABAH** (2 μM) with addition of $ONOO^-$ (4 μM) (inset) followed by the addition of GSH (0 – 2 μM), and 1 min wait in buffer solution [8% DMSO, 1 mM CTAB] (pH = 8.20 at 25 ^oC) Fluorescence intensities were measured with λ_{ex} = 390 nm/ λ_{em} = 451 nm with slit widths ex slit: 4 nm and em slit: 4 nm.

With **GSH-ABAH** in hand, we evaluated the changes in the UV-Vis absorption of **GSH-ABAH** in the presence of both GSH and $ONOO^-$. The maximum absorption of **GSH-ABAH** at 326 nm shifted to 370 nm with the addition of $ONOO^-$ while the absorption peak does not change with addition of GSH, which is consistent with the PET process (Fig S1-2). Fluorescence experiments with $ONOO^-$ were then carried out. As shown in Fig. 2 and S3, **GSH-ABAH** was initially non-fluorescent, however upon the addition of $ONOO^-$ (4 μM), a small fluorescence increase was observed. However, a large increase in fluorescence intensity (> 10-fold, see Fig. 2 and S4) was then observed following the subsequent addition of GSH (0 – 2 μM). This observation demonstrated the requirement of both $ONOO^-$ 'AND' GSH to obtain a significant turn "on" fluorescence response.

The addition of both analytes was then carried out in reverse order. Similarly, the addition of GSH (5 μM) only resulted in a small increase in fluorescence intensity (Fig. 3 and S5). However, as expected a large fluorescence increase was observed after the subsequent addition of $ONOO^-$ (0 - 14 μM) (Fig. 3 and Fig. S6). In order to confirm the reaction mechanism,

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HRMS experiments were carried out. Initially, the probe GSH-ABAH was dissolved in acetonitrile and the mass spectra was obtained (Fig. S11). Then 2 eq of ONOO- (in water) was added to the solution of GSH-ABAH and the mass spectra observed was consistent with deprotection of the phenol (Fig. S12 and Scheme 1). Subsequently, 1 eq GSH (in water) was added to the solution and a mass peak at 630.1554 was observed confirming the reaction of GSH with the maleic anhydride group *via* electrophilic addition (Fig. S13 and Scheme 1). These results clearly demonstrate the ability of **GSH-ABAH** to perform AND-logic with $ONOO^-$ and GSH.

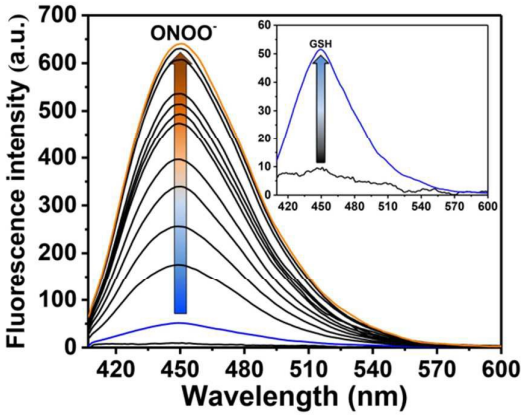

Fig. 3. - Fluorescence spectra of **GSH-ABAH** (2 μM) with addition of GSH (5 μM), 1 min wait (inset), then addition of $ONOO^-$ (0 – 14 μM) in buffer solution [8% DMSO, 1 mM CTAB] (pH = 8.20 at 25 °C) Fluorescence intensities were measured with λ_{ex} = 390 nm/ λ_{em} = 451 nm with slit widths ex slit: 4 nm and em slit: 4 nm.

Next, we evaluated the selectivity of probe **GSH-ABAH** towards a number of different biologically relevant amino acids including serine, lysine and methionine (Fig. S7). The amino acids without a thiol (S-H) group led to no change in fluorescence intensity of **GSH-ABAH**. However, as predicted, thiol (S-H) containing biological analytes (Glutathione, Cystine and Homocystine) induced an enhancement in fluorescence intensity. While **GSH-ABAH** demonstrated an excellent selectivity for $ONOO^-$ over reactive oxygen/nitrogen species including H_2O_2 (Fig. S8).

We then carried out kinetic studies for **GSH-ABAH** with both $ONOO^-$ and GSH (Fig. S9, S10). After initial addition of GSH or $ONOO^-$, followed by the subsequent addition of the second analyte a significant increase in fluorescence within 30s was observed. Furthermore, HRMS experiments confirmed the proposed reaction mechanism (Fig S11, S12 and S13).

Due to these results, **GSH-ABAH** was then evaluated for cellular imaging of GSH and $ONOO^-$. RAW264.7 cells were pre-treated with N-ethylmaleinide (NEM, GSH scavenger) before incubation with **GSH-ABAH**. Subsequently, GSH or SIN-1 (a peroxynitrite donor)[15] were added to produce intracellular GSH or $ONOO^-$. As shown in Fig. 4 and S14, the addition of GSH or $ONOO^-$ led to no fluorescence response in cells. However, treatment of RAW264.7 cells with both GSH and SIN-1 resulted in a significant increase in the fluorescence intensity enabling the visualisation of both species in living cells.

In summary, we have developed an ESIPT-based 'AND' logic fluorescence probe (**GSH-ABAH**) for the detection of $ONOO^-$ and biological thiols. **GSH-ABAH** was shown to have high sensitivity and selectivity towards $ONOO^-$ and biothiols. More importantly, **GSH-ABAH** was able to visualise exogenous $ONOO^-$ and GSH in RAW264.7 cells. This simple novel 'AND' logic-based system provides a scaffold for the further development of a multi-analyte probes. We are now turning our attention to the development of longer wavelength ESIPT-based probes for multi-analyte *in-vivo* imaging.

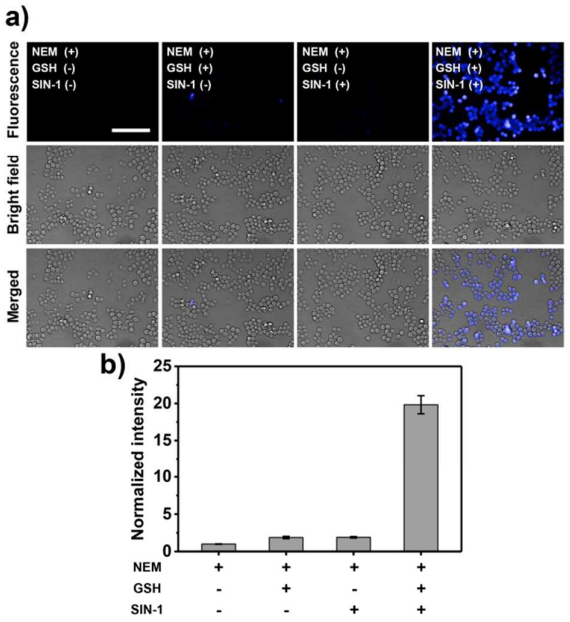

Fig. 4 Fluorescence imaging (a) and quantification (b) of RAW264.7 cells with **GSH-ABAH** (20 μM) in the presence of exogenously added GSH (300 μM) and/or SIN-1 (500 μM) with 1% DMSO. Excitation channel 360–400 nm, emission channel filtered = 410-480 nm. Scale bar = 100 μm. Error bars represent SD. Note: The cells were pre-incubated with N-ethylmaleimide (NEM, GSH scavenger)

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Conflicts of interest

No conflicts of interest

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