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 permeability 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 species¹ 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.⁴⁵ In addition, biological thiols such as glutathione (GSH) and cysteine (Cys) are essential in maintaining biological redox homeostasis.⁶⁷ GSH is a natural tripeptide (γ-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.¹²¹⁴ 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.⁷¹⁶¹⁷

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 ions²² and reactive oxygen species²³²⁴ 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.²⁶²⁷

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 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,²⁹³⁰ despite numerous fluorescence based probes being developed for the sensing of these analytes separately.³¹³² 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 excited state 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

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 and Scheme S1, ESI†). 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 H₂O₂,42 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 and 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 5-aminosalicylic acid in polyphosphoric acid (PPA) at 180 °C. 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 K₂CO₃ in DMF to afford GSH-ABAH in 27% yield (Scheme S2, ESI†). The chemical structure of GSH-ABAH was fully characterized by ¹H NMR, ¹³C NMR and high resolution mass spectrometry (HRMS).

We then 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 and S2, ESI†). Fluorescence experiments with ONOO⁻ were then carried out. As shown in Fig. 2 and Fig. S3 (ESI†), 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 Fig. S4, ESI†) 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 Fig. S5, ESI†). However, as expected a large fluorescence increase was observed after the subsequent addition of ONOO⁻ (0–14 µM) (Fig. 3 and Fig. S6, ESI†).
Next, we evaluated the selectivity of probe GSH-ABAH towards a number of biologically relevant amino acids including serine, lysine and methionine (Fig. S7, ESI†). 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 H2O2 (Fig. S8, ESI†).

We then carried out kinetic studies for GSH-ABAH with both ONOO− and GSH (Fig. S9 and S10, ESI†). After initial addition of GSH or ONOO−, followed by the subsequent addition of the second analyte a significant increase in fluorescence within 30 s was observed. HRMS experiments were performed, in order to confirm the reaction mechanism. When 2 eq. of ONOO− (in water) was added to a solution of GSH-ABAH (HRMS in acetonitrile Fig. S11, ESI†) the mass spectra was consistent with deprotection of the phenol (Fig. S12 (ESI†) and Scheme 1). Subsequently, 1 eq. GSH (in water) was added a mass peak at 630.1354 was observed confirming the reaction of GSH with the maleic anhydride group via electrophilic addition (Fig. S13 (ESI†) and Scheme 1). These results clearly demonstrate the ability of GSH-ABAH to perform ‘AND’ logic with ONOO− ‘AND’ GSH.

Due to these results, GSH-ABAH was then evaluated for cellular imaging of GSH and ONOO−. RAW264.7 cells were pre-treated with N-ethylmaleimide (NEM, GSH scavenger) before incubation with GSH-ABAH. Subsequently, GSH or SIN-1 (a peroxynitrite donor)15 were added to produce intracellular GSH before incubation with GSH-ABAH.

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).

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.

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**Conflicts of interest**

No conflicts of interest.