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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 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 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]

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,[29,30] despite numerous fluorescence based probes being developed for the sensing of these analytes separately.[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 excited state intramolecular proton transfer (ESIPT) 'AND' logic gate for the simultaneous detection of $ONOO^-$ 'AND' GSH. Owing to the attractive characteristics of

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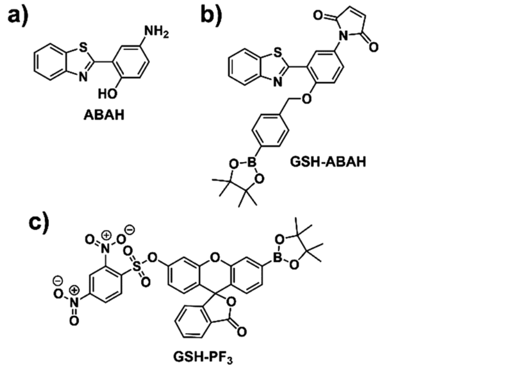


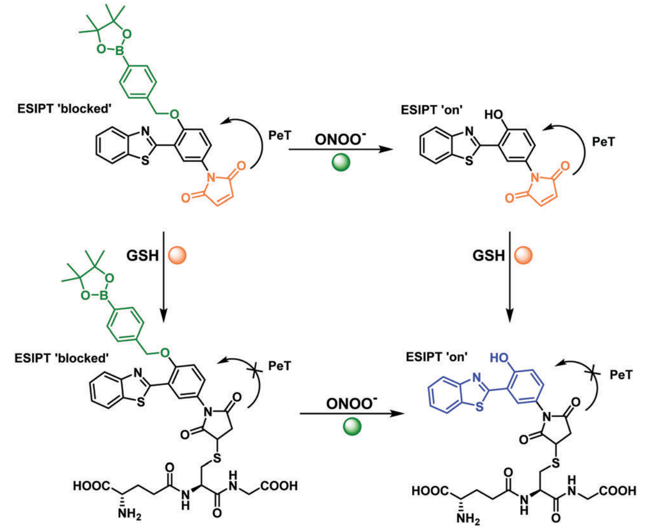
Fig. 1 (a) **ABAH** ESIPT fluorophore previously used in the literature (b) this work – ESIPT-based probe **GSH-ABAH** for the detection of $ONOO^-$ and biological thiols (c) structure of the **GSH-PF3** probe previously used for the simultaneous detection of $ONOO^-$ and GSH.

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†).[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 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-aminothiophenol 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 K_2CO_3 in DMF to afford **GSH-ABAH** in 27% yield (Scheme S2, ESI†). The chemical structure of **GSH-ABAH** was fully characterized by ^1H NMR, ^{13}C 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



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

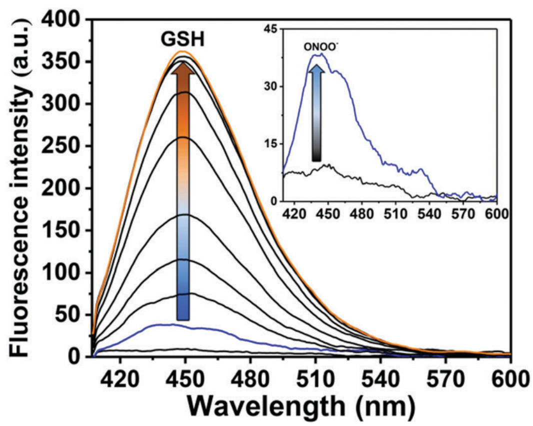


Fig. 2 Fluorescence spectra of **GSH-ABAH** (2 μM) with addition of $ONOO^-$ (4 μM) (inset) followed by the addition of GSH (0–2 μM), and 1 min wait in buffer solution [8% DMSO, 1 mM CTAB] (pH = 8.20 at 25 °C) fluorescence intensities were measured with λ_{ex} = 390 nm/λ_{em} = 451 nm with slit widths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

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†).

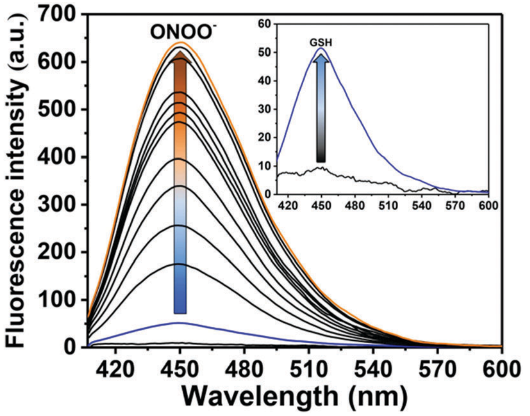


Fig. 3 Fluorescence spectra of **GSH-ABAH** (2 μM) with addition of GSH (5 μM), 1 min wait (inset), then addition of $ONOO^-$ (0–14 μM) in buffer solution [8% DMSO, 1 mM CTAB] (pH = 8.20 at 25 °C) fluorescence intensities were measured with λ_{ex} = 390 nm/λ_{em} = 451 nm with slit widths ex slit: 4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

Next, we evaluated the selectivity of probe **GSH-ABAH** towards a number of 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 H_2O_2 (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 or $ONOO^-$. As shown in Fig. 4 and Fig. S14 (ESI†), the addition of GSH or $ONOO^-$ led to no fluorescence response in cells. However, treatment 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,

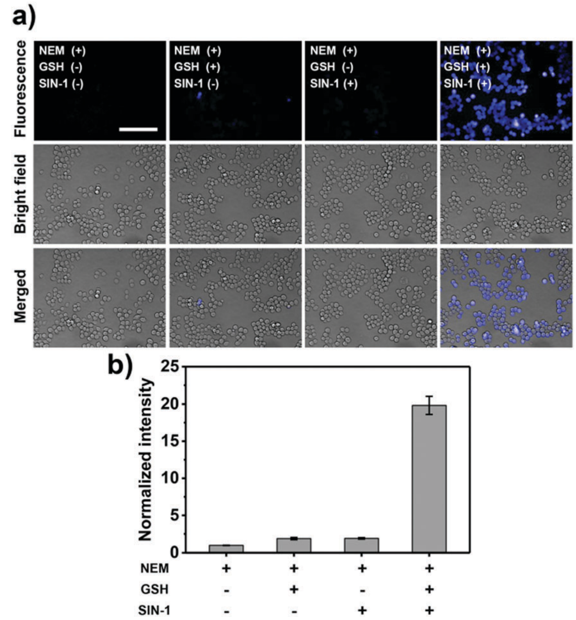


Fig. 4 Fluorescence imaging (a) and quantification (b) of RAW264.7 cells with **GSH-ABAH** (20 μM) in the presence of exogenously added GSH (300 μM) and/or SIN-1 (500 μM) with 1% DMSO. Excitation channel 360–400 nm, emission channel filtered = 410–480 nm. Scale bar = 100 μm. Error bars represent SD.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

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.

Notes and references

1 C. Szabó, H. Ischiropoulos and R. Radi, *Nat. Rev. Drug Discovery*, 2007, **6**, 662.
2 G. Ferrer-Sueta and R. Radi, *ACS Chem. Biol.*, 2009, **4**, 161–177.
3 P. Ascenzi, A. di Masi, C. Sciorati and E. Clementi, *BioFactors*, 2010, **36**, 264–273.
4 H. Ischiropoulos and J. S. Beckman, *J. Clin. Invest.*, 2003, **111**, 163–169.
5 P. Sarchielli, F. Galli, A. Floridi, A. Floridi and V. Gallai, *Amino Acids*, 2003, **25**, 427–436.
6 D. A. Wink, Y. Vodovotz, J. Laval, F. Laval, M. W. Dewhirst and J. B. Mitchell, *Carcinogenesis*, 1998, **19**, 711–721.
7 P. Pacher, J. S. Beckman and L. Liaudet, *Physiol. Rev.*, 2007, **87**, 315–424.
8 J. S. Stamler and A. Slivka, *Nutr. Rev.*, 1996, **54**, 1–30.
9 S. Iwata, T. Hori, N. Sato, Y. Ueda-Taniguchi, T. Yamabe, H. Nakamura, H. Masutani and J. Yodoi, *J. Immunol.*, 1994, **152**, 5633–5642.
10 H. Nakamura, K. Nakamura and J. Yodoi, *Annu. Rev. Immunol.*, 1997, **15**, 351–369.
11 G. Wu, Y.-Z. Fang, S. Yang, J. R. Lupton and N. D. Turner, *J. Nutr.*, 2004, **134**, 489–492.
12 S. C. Lu, *Mol. Aspects Med.*, 2009, **30**, 42–59.
13 S. Seshadri, A. Beiser, J. Selhub, P. F. Jacques, I. H. Rosenberg, R. B. D'agostino, P. W. Wilson and P. A. Wolf, *N. Engl. J. Med.*, 2002, **346**, 476–483.
14 D. M. Townsend, K. D. Tew and H. Tapiero, *Biomed. Pharmacother.*, 2003, **57**, 145–155.
15 M. Balazy, P. M. Kaminski, K. Mao, J. Tan and M. S. Wolin, *J. Biol. Chem.*, 1998, **273**, 32009–32015.
16 K.-A. Marshall, M. Reist, P. Jenner and B. Halliwell, *Free Radical Biol. Med.*, 1999, **27**, 515–520.
17 J. P. Bolaños, S. J. Heales, J. M. Land and J. B. Clark, *J. Neurochem.*, 1995, **64**, 1965–1972.
18 A. C. Sedgwick, J. E. Gardiner, G. Kim, M. Yevglevskis, M. D. Lloyd, A. T. A. Jenkins, S. D. Bull, J. Yoon and T. D. James, *Chem. Commun.*, 2018, **54**, 4786–4789.
19 M. L. Odyniec, A. C. Sedgwick, A. H. Swan, M. Weber, T. S. Tang, J. E. Gardiner, M. Zhang, Y.-B. Jiang, G. Kociok-Kohn, R. B. Elmes, S. D. Bull, X.-P. He and T. D. James, *Chem. Commun.*, 2018, **54**, 8466–8469.
20 L. Wu, Q. Yang, L. Liu, A. C. Sedgwick, A. J. Cresswell, S. D. Bull, C. Huang and T. D. James, *Chem. Commun.*, 2018, **54**, 8522–8525.
21 L. Wu, Y. Wang, M. Weber, L. Liu, A. C. Sedgwick, S. D. Bull, C. Huang and T. D. James, *Chem. Commun.*, 2018, **54**, 9953–9956.
22 D. Wu, L. Chen, W. Lee, G. Ko, J. Yin and J. Yoon, *Coord. Chem. Rev.*, 2018, **354**, 74–97.
23 N. Soh, *Anal. Bioanal. Chem.*, 2006, **386**, 532–543.
24 X. Jiao, Y. Li, J. Niu, X. Xie, X. Wang and B. Tang, *Anal. Chem.*, 2017, **90**, 533–555.
25 (*a*) D. Wu, A. C. Sedgwick, T. Gunnlaugsson, E. U. Akkaya, J. Yoon and T. D. James, *Chem. Soc. Rev.*, 2017, **46**, 7105–7123; (*b*) X.-P. He and H. Tian, *Chem*, 2018, **4**, 246–268; (*c*) Y. Fu, H.-H. Han, J. Zhang, X.-P. He, B. L. Feringa and H. Tian, *J. Am. Chem. Soc.*, 2018, **140**, 8671–8674; (*d*) J. Zhang, Y. Fu, H.-H. Han, Y. Zang, J. Li, X.-P. He, B. L. Feringa and H. Tian, *Nat. Commun.*, 2017, **8**, 987.
26 S. Erbas-Cakmak, S. Kolemen, A. C. Sedgwick, T. Gunnlaugsson, T. D. James, J. Yoon and E. U. Akkaya, *Chem. Soc. Rev.*, 2018, **47**, 2228–2248.
27 J. L. Kolanowski, F. Liu and E. J. New, *Chem. Soc. Rev.*, 2018, **47**, 195–208.
28 (*a*) A. Romieu, *Org. Biomol. Chem.*, 2015, **13**, 1294–1306; (*b*) X.-P. He, X.-L. Hu, T. D. James, J. Yoon and H. Tian, *Chem. Soc. Rev.*, 2017, **46**, 6687–6696.
29 F. Yu, P. Li, G. Li, G. Zhao, T. Chu and K. Han, *J. Am. Chem. Soc.*, 2011, **133**, 11030–11033.
30 F. Yu, P. Li, B. Wang and K. Han, *J. Am. Chem. Soc.*, 2013, **135**, 7674–7680.
31 S. Wang, L. Chen, P. Jangili, A. Sharma, W. Li, J.-T. Hou, C. Qin, J. Yoon and J. S. Kim, *Coord. Chem. Rev.*, 2018, **374**, 36–54.
32 H. S. Jung, X. Chen, J. S. Kim and J. Yoon, *Chem. Soc. Rev.*, 2013, **42**, 6019–6031.
33 A. C. Sedgwick, H.-H. Han, J. E. Gardiner, S. D. Bull, X.-P. He and T. D. James, *Chem. Sci.*, 2018, **9**, 3672–3676.
34 C. B. Pocernich and D. A. Butterfield, *Biochim. Biophys. Acta, Mol. Basis Dis.*, 2012, **1822**, 625–630.
35 V. V. Shynkar, A. S. Klymchenko, C. Kunzelmann, G. Duportail, C. D. Muller, A. P. Demchenko, J. M. Freyssinet and Y. Mely, *J. Am. Chem. Soc.*, 2007, **129**, 2187–2193.
36 M. Santra, B. Roy and K. H. Ahn, *Org. Lett.*, 2011, **13**, 3422–3425.
37 J. E. Kwon and S. Y. Park, *Adv. Mater.*, 2011, **23**, 3615–3642.
38 K. S. Hwang, K. Y. Park, D. B. Kim and S.-K. Chang, *Dyes Pigm.*, 2017, **147**, 413–419.
39 Y. Zhao, Y. Xue, H. Li, R. Zhu, Y. Ren, Q. Shi, S. Wang and W. Guo, *Spectrochim. Acta, Part A*, 2017, **175**, 215–221.
40 H. Yao and T. Funada, *Chem. Commun.*, 2014, **50**, 2748–2750.
41 L. Cui, W. Zhu, Y. Xu and X. Qian, *Anal. Chim. Acta*, 2013, **786**, 139–145.
42 A. Sikora, J. Zielonka, M. Lopez, J. Joseph and B. Kalyanaraman, *Free Radical Biol. Med.*, 2009, **47**, 1401–1407.
43 T. Matsumoto, Y. Urano, T. Shoda, H. Kojima and T. Nagano, *Org. Lett.*, 2007, **9**, 3375–3377.