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ESIPT-based fluorescence probe for the rapid detection of hypochlorite (HOCl/ClO^-)†

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ESIPT-based fluorescence probes are emerging as an attractive tool for the detection of biologically relevant analytes owing to their unique photophysical properties. In this work, we have developed an ESIPT-based fluorescence probe (TCBT-OMe) for the detection of HClO/ClO^- through the attachment of a bioorthogonal dimethylthiocarbamate linker. TCBT-OMe was shown to rapidly detect HClO/ClO^- (<10 s) at biologically relevant concentrations (LoD = 0.16 nM) and have an excellent selectivity towards others ROS/RNS and amino acids. Therefore, TCBT-OMe was tested in live cells and was successfully shown to be able to detect endogenous and exogenous HClO/ClO^- in HeLa cells. Additionally, TCBT-OMe acts as a dual input logic gate for Hg^{2+} and H_2O_2. Interestingly, Hg^{2+} alone gradually causes a fluorescence response but requires >30 min to produce a fluorescence response. Test strips containing TCBT-OMe were prepared and were demonstrated as an effective way to detect HClO/ClO^- in water. Furthermore, TCBT-OMe was shown to detect exogenously added HClO/ClO^- in three different water samples with little interference thus demonstrating the effectiveness as a method for the detection of HClO/ClO^- in drinking water samples.

Hypochlorous acid (HOCl) is a biologically important reactive oxygen species (ROS), which partially dissociates to form its hypochlorite anion (ClO^-) under physiological conditions. In biological systems, myeloperoxidase, an enzyme found in leukocytes produces HOCl/ClO^- by catalysing the reaction between $Cl + H_2O_2 \rightarrow HOCl$.[1] This vital ROS is used in immune defence systems due to its microbicidal properties.[1] Unfortunately, excessive production of HOCl/ClO^- can lead to the damage of a range of biological targets such as amino acids, proteins, carbohydrates and lipids.[2,3] As a consequence, HOCl/ClO^- has been associated with a number of diseases causing cell and tissue damage.[4]

In addition to its role in biological systems, HOCl/ClO^- is produced by the chlorination of water ($Cl_2 + H_2O \rightarrow HOCl$), which is the most common method for the treatment of water especially in public swimming pools.[5] NaOCl (Bleach) is also extensively used as a disinfectant for both domestic and industrial purposes. Unfortunately, over-exposure to HOCl/ClO^-, results in swimming pool-associated asthma, irritation to the oesophagus, throat and spontaneous vomiting (http://www.who.int/water_sanitation_health/dwq/chlorine.pdf).[6] Additionally, there is an increased risk of bladder cancer associated with chlorinated by-products produced from chlorinated water.[7,8] Therefore, given the potential health hazard towards animals and humans, the development of an effective method for HOCl/ClO^- detection is required.

Within our research group, we are interested in developing reaction-based fluorescence sensors for the detection of biologically important analytes.[9–13] Small-molecule fluorescence probes are a particular attractive tool owing to their high sensitivity, selectivity and high spatial and temporal resolution.[14] In particular, we are interested in using Excited State Intramolecular Proton Transfer (ESIPT)-based fluorescence probes due to their excellent photophysical properties, which include intense luminescence, photostability and a large stokes shift.[15,16] Previously, we reported an ESIPT-based fluorescence probe for the detection of peroxynitrite ($ONOO^-$) through the use of a benzyl boronic ester protecting group (Scheme 1).[15] This protecting group blocked the ESIPT process and therefore a low fluorescence intensity was observed. The addition of $ONOO^-$, resulted in the fluorophore's deprotection and an increase in fluorescence intensity was observed.

In this work, we believed a methoxy-hydroxybenzothiazole (**HBT-OMe**) fluorophore would provide an effective ESIPT fluorescence probe for the detection of HOCl/ClO^- (see ESI,† S1).[17,18]

To obtain **TCBT-OMe** we first prepared **HBT-OMe** by the addition of a 2 : 1 H_2O_2–(30% in H_2O)/HCl solution to 2-aminothiophenol

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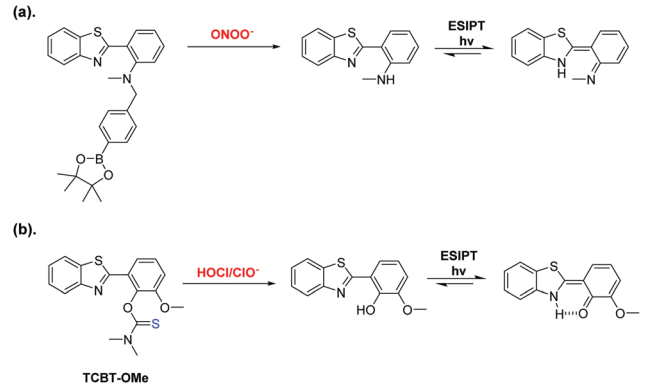

Scheme 1 (a) Our previously reported ESIPT probe for the detection of $ONOO^-$. (b) This work – a thiocarbamate linker-based ESIPT **TCBT-OMe** for the detection of $HOCl/ClO^-$.

and *O*-vanillin in EtOH. This reaction proceeded quickly and smoothly, in a good yield (68%). With **HBT-OMe** in hand, four equivalents of dimethylthiocarbamoyl chloride was then added slowly to a solution of **HBT-OMe** in DCM. DIPEA was subsequently added dropwise to the reaction, which produced **TCBT-OMe** in excellent yield (72%).

We then evaluated the UV-Vis of **TCBT-OMe** with the addition of $HOCl/ClO^-$ (10 μM), which resulted in the formation of a UV absorption peak at ~310 nm (see ESI,† Fig. S1). Bhattacharyya *et al.* have reported that the fluorescence emission of the ESIPT process can be effected by intermolecular hydrogen bonding.[19,20] Therefore, evaluation of ESIPT-based fluorescence probes are commonly carried out in the presence of the surfactant cetyl trimethylammonium bromide (CTAB, 1 mM) or by using a large ratio of organic solvent.[19,21–23] It is believed that the formation of a micellar environment creates a hydrophobic pocket that aids the ESIPT process. Therefore, we evaluated the ability of **TCBT-OMe** to detect $HOCl/ClO^-$ by fluorescence in the presence of CTAB, 1 mM. As shown in Fig. 1a, **TCBT-OMe** was found to be very sensitive towards $HOCl/ClO^-$ reacting with micromolar concentrations to produce a large increase in fluorescence (~42 fold – Fig. S3, ESI†). **TCBT-OMe** was shown to rapidly react with $HOCl/ClO^-$ producing a fluorescence response within less than 10 s (see ESI,† Fig. S4) and have a very low Limit of Detection (LoD) of 0.16 nM (see ESI,† Fig. S5). $HOCl/ClO^-$ (35 μM) was added to **TCBT-OMe** at different pH values and a bell-shaped curve was observed. The largest fluorescence response was seen at the pK_a of $HOCl/ClO^-$ = 7.53 (Fig. S5, ESI†) suggestive of general acid–base catalysis being in operation. (see ESI,† Scheme S1 for proposed mechanism).

We then evaluated the selectivity of **TCBT-OMe** towards other reactive oxygen/nitrogen species (ROS/RNS) and amino acids (Fig. 1b). Remarkably, **TCBT-OMe** had an excellent selectivity towards $HOCl/ClO^-$ therefore permitting its use as a fluorescence probe for the detection of $HOCl/ClO^-$ in live cells. As shown in Fig. 2, **TCBT-OMe** was successfully used to visualise endogenously stimulated $HOCl/ClO^-$ in HeLa cells using phorbol 12-myristate 13-acetate (**PMA**, which is a ROS stimulant that induces the production of $HOCl/ClO^-$). Separately, HeLa cells were

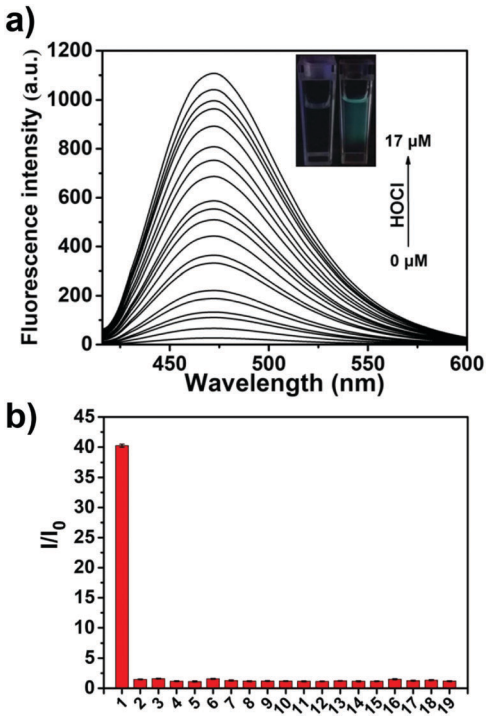

Fig. 1 (a) Fluorescence spectra of **TCBT-OMe** (5 μM) with increasing additions of $HOCl/ClO^-$ (from 0 to 17 μM) in PBS buffer (pH 7.4, containing 1% DMSO, 1 mM CTAB). Measurements were taken after 1 min. λ_{ex} = 310 nm. Slit widths: ex = 6 nm em = 4 nm. (b) Selectivity bar chart of **TCBT-OMe** in PBS pH 7.4, containing 1% DMSO, 1 mM CTAB with HClO (15 μM) and other interfering reagents (ROS/RNS and various amino acids). 1, HClO; 2, blank; 3, $ONOO^-$; 4, H_2O_2; 5, $ROO^\bullet$; 6, $^\bullet OH$; 7, $^\bullet O_2^-$; 8, $^1O_2$; 9, NO; 10, glycine; 11, asparagine; 12, cysteine; 13, homocysteine; 14, glutathione; 15, arginine; 16, histidine; 17, serine; 18, glycine; 19, threonine. Note: the concentration of **TCBT-OMe** and each interfering species are 5 μM and 100 μM respectively, 30 min wait before measurement in buffer solution. λ_{ex} = 310 nm/λ_{em} = 472 nm error bars represent s.d. Measurements were taken after 30 min. λ_{ex} = 310 nm. Slit widths: ex = 6 nm, em = 4 nm.

also pretreated with 4-aminobenzoic acid hydrazide (**ABAH**, which is a specific inhibitor of MPO which suppressed the generation of HOCl) and as expected only weak fluorescence was observed. **TCBT-OMe** was also able to detect $HOCl/ClO^-$ added exogenously to the HeLa cells.

The dimethylthiocarbamate linker of **TCBT-OMe** has previously been used in the construction of dual input molecular logic gate[24] for the detection of Hg^{2+} 'AND' H_2O_2 (see ESI† Scheme S2 for proposed mechanism).[25,26] Therefore, we evaluated the ability of **TCBT-OMe** to perform molecular logic with the input of Hg^{2+} and H_2O_2. The presence of solely H_2O_2 (120 μM) led to a small increase in fluorescence intensity (dashed line), however, with subsequent additions of Hg^{2+} (0–9 μM) a large fluorescence response was observed (Fig. 3a). To demonstrate that both analytes are required, Hg^{2+} was added first, followed by the addition of H_2O_2 (0–180 μM). As shown in Fig. 3b, the subsequent addition of H_2O_2 rapidly led to an increase in fluorescence intensity. **TCBT-OMe** was shown to be selective towards Hg^{2+} over other metal cations in the presence of H_2O_2 (see ESI,† Fig. S9). Interestingly, Hg^{2+} alone resulted in a slow increase in fluorescence intensity (see ESI,† Fig. S10). This is believed to be

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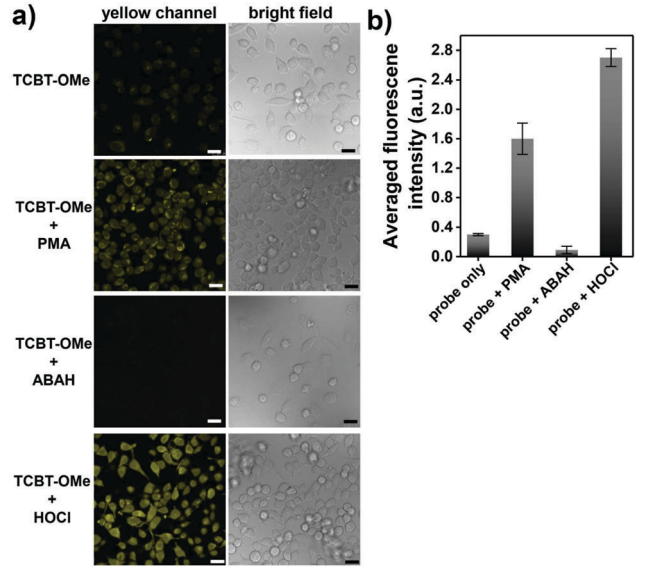

Fig. 2 (a) From top to bottom: HeLa cells were pretreated with **TCBT-OMe** (40 μM) for 30 min; HeLa cells pretreated with **TCBT-OMe** (40 μM) were then left for 30 min after preincubation with PMA (1.2 μg mL^{-1}) for 90 min; HeLa cells pretreated with **TCBT-OMe** (40 μM) were then left for 30 min after preincubation with 250 μM ABAH for 70 min; HeLa cells loaded with **TCBT-OMe** (40 μM) for 30 min followed by the exogenous addition of 8 μM NaOCl for 5 min. Scale bar: 25 μm λ_{ex} = 420 nm/λ_{em} = 420–590 nm. (b) The histogram shows the semi-quantitative calculation of averaged fluorescence intensity (FI) of each fluorescence panel in the displayed images by ImageJ software.

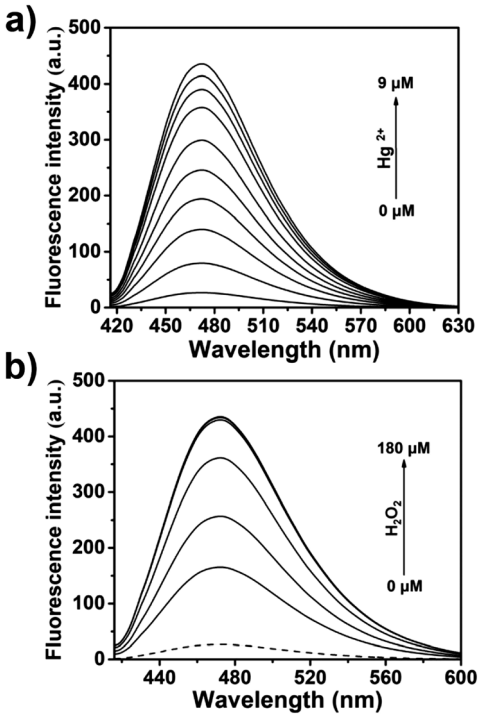

Fig. 3 (a) Fluorescence spectra of **TCBT-OMe** (5 μM) in the presence of H_2O_2 (120 μM) – (dashed line represent probe and H_2O_2) with increasing concentrations of Hg^{2+} (0–9 μM) in buffer solution pH 7.4, 1% DMSO, 1 mM CTAB 14 min wait between measurement. λ = 310 nm. Slit widths: ex = 6 nm em = 4 nm. (b) Fluorescence spectra of **TCBT-OMe** (5 μM) in the presence of Hg^{2+} (9 μM) – (dashed line represents probe and Hg^{2+}) with increasing concentrations of H_2O_2 (final concentration: 0, 20, 40, 80, 100, 120, 140 μM and 180 μM) in PBS pH 7.4, containing 1% DMSO, 1 mM CTAB. 14 min wait between measurement in buffer solution. λ_{ex} = 310 nm. Slit widths: ex = 6 nm em = 4 nm.

due to the instability of the dimethylcarbonate formed from the reaction of **TCBT-OMe** with Hg^{2+}.

Despite this interesting dual responsive reactivity of **TCBT-OMe**, this 'AND' logic requires minutes to fully react, whereas HOCl/ClO^- reacts with **TCBT-OMe** within seconds. Therefore, due to the significantly greater reactivity of **TCBT-OMe** towards HOCl/ClO^- over Hg^{2+}, we believed we could use it as an effective method for the detection of HOCl/ClO^- in drinking water sources.

We produced test strips by simply soaking a commercially available test strip in water containing **TCBT-OMe** (0.8 mM). After drying, test strips impregnated with **TCBT-OMe** were placed in water containing HClO/ClO^- (0–200 μM). As shown in Fig. 4, there is a clear colour/intensity difference in the test strips that have been dipped into water containing various concentrations of HClO/ClO^-.

In addition to detecting HClO/ClO^- in water, **TCBT-OMe** was added into three different water samples containing 1 mM CTAB (Sample A, tap water from University of Bath; Sample B, water from the Avon River (Bath); Sample C, water from Roman spa in Bath). Interestingly, little interference was observed for the exogenous addition of HClO/ClO^- to each water sample (>95% recovery) – see ESI,† Table S1.

In summary, we have developed an ESIPT-based fluorescence **TCBT-OMe** for the detection of HClO/ClO^-. **TCBT-OMe** was shown to have a very high sensitivity and selectivity towards HClO/ClO^- fully reacting within 10 s and having a LoD of 0.16 μM. Significantly, **TCBT-OMe** was able to detect endogenous and

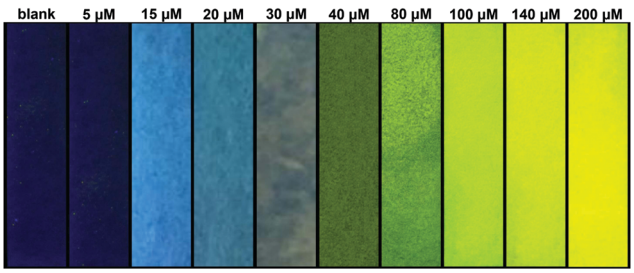

Fig. 4 Photograph showing the colour changes of **TCBT-OMe** impregnated test strips after addition to water samples containing different concentrations of HClO/ClO^- under UV light (365 nm).

exogenous HClO/ClO^- in HeLa cells. Additionally, **TCBT-OMe** was shown as a dual input logic gate with Hg^{2+} and H_2O_2 as inputs. Interestingly, Hg^{2+} alone gradually produced a fluorescence response but required >30 min to produce a significant fluorescence response. Test strips containing **TCBT-OMe** were developed and shown to be an effective way to detect HClO/ClO^- in water. Furthermore, **TCBT-OMe** was shown to detect exogenously added HClO/ClO^- in three different water samples with little interference demonstrating its effectiveness as a method to detect HClO/ClO^- in drinking water samples.

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Conflicts of interest

No conflicts of interest.

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