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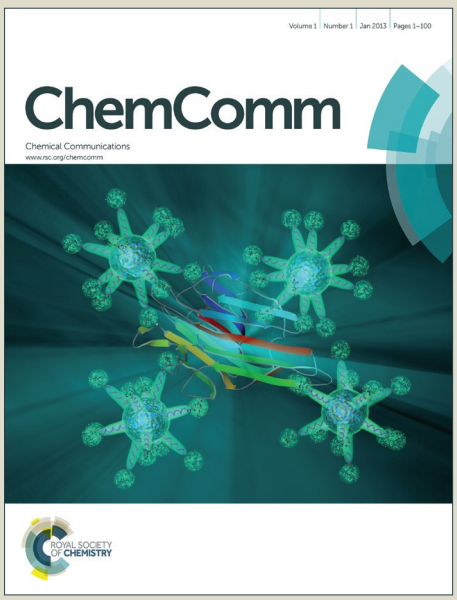

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A cyclization-induced emission enhancement (CIEE)-based ratiometric fluorogenic and chromogenic probe for the facile detection of a nerve agent simulant DCP

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The first ratiometric fluorescent probe for detection of a nerve agent stimulant was developed based on tandem phosphorylation and intramolecular cyclization, by which high sensitivity as well as large emission shift could be achieved.

Nerve agents (NA) are highly toxic volatile liquid, gas or aerosols that irreversibly block the enzyme acetylcolinesterase in the neuronal synapses, thus disrupting nerve impulse transmission which causes severe effects on human and animal health leading to death through the paralysis of respiratory muscles.[1] These compounds are a family of highly toxic phosphoric acid esters, structurally related to the larger family of organophosphate (OP) compounds. Nerve gas agents can be classified according to the following criteria:1) Boiling point: Nerve agents can be classified into two categories namely volatile and non volatile. 2) Country of origin: Agents originally developed in Germany were designated as "G series" agents; these include Sarin (GB), Tabun (GA) and Soman (GD). 3) Toxicity: "V series" agents, V stand for venomous and include VE, VG, and VX.

Organophosphorus compounds (OPs) are also key components in agricultural pesticides and herbicides, which play vital roles in modern agriculture. However, the extensive overuse of these compounds has led to the poisoning of thousands of humans throughout the world. These OP compounds are structurally similar to nerve gases and are also acetylcholinesterase inhibitors. As a consequence, there is a need to develop sensitive and selective receptors for the detection of said compounds. Techniques used to detect organophosphorus (OPs) and chemical warfare agents (CWAs) include, electrical sensors,[2] surface acoustic waves,[3] calorimetric methods,[4] electrochemistry,[5] lanthanide luminescence,[6] photonic crystals[7] and fluorescent indicators.[8] Chromo-fluorogenic sensors are attractive as a result of their excellent operational simplicity, portability, cost effectiveness and power of real time detection and the ability to integrate functionality that reacts with CWAs. There are many different analytical techniques developed based on changes in the fluorescence properties of a molecule in different environments. The recently developed methods are PET based probes,[9]cyclisation reactions[10] in push–pull chromophores, displacement like assays,[11] molecular impregnated polymers,[12] oximate containing sensors,[13] nucleophilic substitution reactions[14] and complex formation based probes.[15]

The general mechanism for chemical probes to detect nerve agents is that nucleophilic probe molecule attacks on the electrophilic phosphorous centre of the nerve agents to form phosphate ester[16], which exactly mimics the reaction of AChE with the nerve agents. The resultant phosphate ester of probe molecule could then undergo intramolecular transformations to generate detectable species. Oximes (R^1R^2C=NOH) include aldoximes and ketoximes in which R^1 is a hydrogen or another organic group.[17] An oxime is a ''super-nucleophile'' and is capable of attacking the internal phosphorus at the phosphorylated serine–OH in AChE to restore the serine–OH, and thus the function of AChE.[18]

Despite these elegant studies, there is still significant room for improvement. In addition to quick response and high sensitivity, a truly practically useful fluorescence probe should display a ratiometric and colorimetric response. Such features can reduce the background interference and false positive results. Moreover, a probe enabling us to analyze samples using easy-to-use devices is highly desired for real time studies. Finally, such a probe should be able to be employed for the analysis of both liquid and gaseous nerve agents.

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Though many existing nucleophilic nerve agent sensors have been reported in the literature (Table S3, ESI†), ring open-close based sensors for 'in-field' application have not been explored so far. Herein, we wish to disclose the first napthothiazolium conjugated benzothiazole derivative (**NTBT**) as the ratiometric fluorogenic and chromogenic probe for detection of diethyl chlorophosphate (DCP), a nerve agent simulant. In considerably high polarity solvent (e.g. DMSO, MeOH etc.) or under the presence of UV light, **NTBT** is converted to the corresponding Zwitterionic form and it was reported to be a new class of fluorescent dyes due to their favourable photophysical properties including an emission maximum beyond 600 nm and a relatively large Stokes shift.

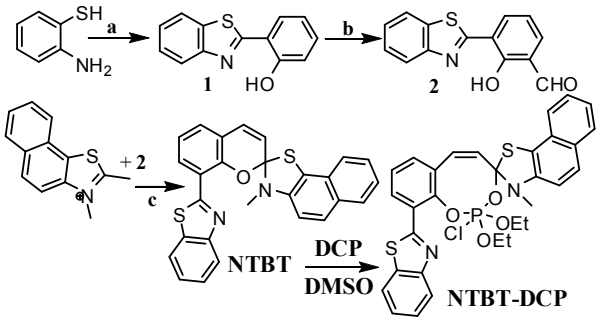
Importantly, this Zwitterionic form of dyes possesses two highly reactive centers such as nucleophilic phenol anion for electrophilic and indolium group for widespread nucleophilic attack[19]. Therefore, it was expected that the reaction of the sensors to electrophilic phosphorous centre of the nerve agents DCP resulted in the phosphorylation of a hydroxyl group. Next, a tandem intramolecular nucleophilic attack of phosphorus oxygen toward the indolium group occurred to generate an eight-membered-ring (Fig S1, ESI†). To the best of our knowledge, this is the first report on a ring open-closed based chemosensor that has instantaneous ratiometric fluorescence response due to cyclization–induced emission enhancement (CIEE) and a nanomolar level detection limit upon exposure to the nerve agent simulants.

Our approach was to create a very simple colorimetric and ratiometric fluorescent sensor system that contains hidden highly nucleophilic and electrophilic centres. The probe **NTBT** displays several notable features; i.e. (i) an amine nitrogen driven *in situ* generation of a highly reactive nucleophilic phenol anion and a potential thiazolium group under UV irradiation or dissolving in high polar solvent (e.g. DMSO). (ii) these reactive handles can react with electrophilic OP nerve agents with fast response (within 1 min), (iii) no base is required for generation of nucleophilic species, (iv) an ICT strategy is implemented by combination of suitable donor-acceptor structures to get ratiometric response, and (v) finally, the probe exhibits noticeable color changes under UV light and even with the naked eye to detect both liquid and gas nerve agents.

The synthesis of chemosensor **NTBT** started with the preparation of hydroxybenzthioimidazole **1**, which was obtained using standard condensation reaction between o-aminothiophenol and salicylaldehyde. Formylation of hydroxybenzthioimidazole **1** afforded the key intermediate, hydroxyaldehyde [20]. With the key intermediate **2** in hand, the stage was set to prepare hybrid chemosensor dye **NTBT** (Scheme 1). Then the probe **NTBT** was synthesized in one step condensation reaction between naphthylthioimidazolium salt with hydroxyaldehyde **2** **NTBT** (Scheme 1). All the precursor and probe **NTBT** molecules were characterized by various analytical and spectral techniques (ESI†).

To test solvent selectivity of the probe molecule, we first examined the sensing potential of probe molecule with DCP (nerve agent mimic) in various solvents (Fig. S19, ESI†). Among these DMF and DMSO were optimized for DCP due to favorable formation of Zwitterionic form in more polar solvent. Therefore, the optimized test solution has been prepared by mixing a NTBT probe (1.0×10^{-7}M) in aqueous DMSO (3:7 v/v) HEPES buffer solution at pH = 7.4, and this has been used for all the studies reported in this paper.

In order to ascertain the reaction of DCP by **NTBT**, absorption titrations were carried out by adding varying concentrations of DCP to a fixed concentration (0.1 μM) of **NTBT**. As shown in Fig. S12a, ESI†, **NTBT** exhibits a main absorption peak at 616 nm, which is ascribed to the typical ICT band in Zwitterionic form of the hybrid thioimidazole-napthothiazoliumhemicyanine dye. Upon addition of DCP (2.0 μM) to a solution of **NTBT**, the absorbance was gradually decreased with a new peak appearing at 429 nm until saturation after 1.2 equiv.; concomitantly, an obvious color change from green to yellow was clearly observed, suggesting that the ICT is turned off due to the phosphorylation of phenoxide ion and nucleophilic attack of P=O toward the indolium group of **NTBT**. In addition, a well defined isosbestic point was also noted at 494 nm, indicative of the formation of the **NTBT**–DCP adduct.



Reagents: (a) Salicylaldehyde/ CdS nano cat./ Methanol/ hv/ rt/ 2 h; (b) hexamine/ toluene-acetic acid/ reflux/ 12 h (c) EtOH, reflux.

Scheme 1 Preparation of **NTBT** followed by the mode of DCP binding.

Next, we investigated the concentration-dependent response in the fluorescence spectra upon incubation of **NTBT** (1.0×10^{-7}M) with OP nerve agents (Fig. S20, ESI†).

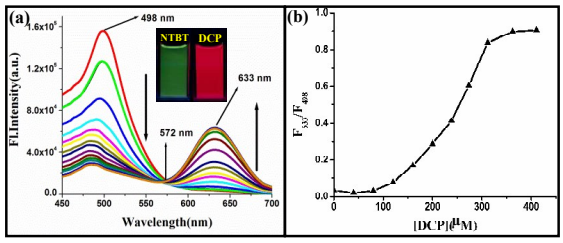


Fig. 1 (a) Emission spectra of **NTBT** (0.1 μM, λ_{ex} = 429 nm) upon addition of DCP (c = 2.0 μM) (b) Fluorescence intensity ratio changes (F_{633}/F_{498}) of **NTBT** (0.1 μM) upon addition of various concentration of DCP.

As shown in Fig. 1a, free probe **NTBT** displayed an emission band centered at 498 nm. After gradual addition of DCP, the emission intensities at 498 nm decreased sharply and concurrently a significant enhancement of emission intensities at 633 nm. Thus, probe **NTBT** exhibited a remarkable emission shift (135 nm) from 498 nm (green) to 633nm (red) as well as a well-defined isoemissive point at 572 nm. These shifts are attributed to the restricted rotation during cyclization via tandem reaction of phosphorylation of the phenoxide with DCP and then intramolecular nucleophilic attack to indolium moiety. We reasoned that the genesis of fluorescence in the assay was achieved via nucleophilic attack of the phenoxide ion of **NTBT** on the phosphate group of DCP, which is concomitant with an intramolecular cyclization. It is worthy to note that such a huge change of signal ratios at two wavelengths is due to CIEE as well as highly desirable for ratiometric fluorescent probe. Furthermore, the fluorescence intensity ratios of probe **NTBT** at 498 and 633 nm (I_{633}/ I_{498}) showed a drastic change from 0.020 to 0.885 upon treatment with DCP (Fig.1b), a 45 fold enhancement in the ratiometric emission. It is worthy to note that such a huge change of signal ratios at two wavelengths is highly desirable for ratiometric fluorescent probes, as the sensitivity and the dynamic range of ratiometric probes are controlled by the ratios. Under the same conditions, the ratio changes of the two fluorescence peaks (I_{633}/ I_{498}) produced an excellent linear function with the concentration of DCP between 0 and 400 μM (Fig. 1b), and the detection limit for DCP was determined as 17 nM based on S/N = 2(Fig. S17 & S18, ESI†). The association constants of DCP with **NTBT** were calculated from UV-vis (= 2.5×10^{7}M) and fluorescence (= 5×10^{7}M) titration experiments by using the Benesi-Hildebrand method(Fig. S15 & S16, ESI†).[21]

To further understand the high selectivity of the probe for DCP over various strong electrophiles like thionyl chloride, phosphorous oxychloride, 4-toluenesulfonyl chloride, acetyl chloride we studied the response of **NTBT** towards these interfering compounds. In all the cases instantaneous colour change was observed but did not lead to any significant ratiometric fluorescence changes (Fig. S22, ESI†).

To understand the absorption and fluorescence properties of **NTBT** and changes of **NTBT** by DCP reaction, density functional theory (DFT) calculations were performed (Table S1&S2 Fig. S23 & S24, ESI†). The TDDFT studies suggest that the vertical major transitions observed at ~590 nm are comparable to those of the experimentally observed spectra at ~616 nm for open **NTBT** isomer(Table S1, ESI†), which is assigned to intramolecular charge transfer (ICT) transition from the benzthioimidazole appended phenoxide to the electron deficient indolyl cation via conjugated sp^2 hybridized C=C bond.This extended conjugation being blocked due to the cyclization with DCP a hypsochromic shift at ~429 nm (theoretically at ~432) in the absorption spectra was observed.

Kinetic investigation of the reaction between **NTBT** and diethyl chlorophosphate showed that the reaction is complete within 40 seconds(Fig. S13a, ESI†).We then examined the kinetic profiles of the reaction under pseudo-first-order conditions with a large excess of DCP (>100 equiv.) over probe **NTBT** (1.0 μM) in aqueous DMSO (3:7 v/v) pH 7.4 HEPES buffer at room temperature and found the pseudo-first-order rate constants for the reaction $k^{/}$ = 0.01 sec^{-1}in all the cases (Fig. S14, ESI†).

The reaction of **NTBT** with DCP was monitored by subjecting the reaction solution to ESI-MS analysis. After treatment of **NTBT** with DCP, the peaks corresponding to the molecular weight of the intermediates and the products were identified; this confirms the formation of these products in assay systems. A major peak located at 623.1213 was identified (Fig. S7 & S9, ESI†), which is in consistence with the theoretical molecular weight of **NTBT**-DCP complex ($C_{31}H_{28}N_2S_2O_4ClP$+; MW: 623.1211), confirming formation of the compound in the assay system.

The reaction between **NTBT** and DCP was also investigated using ^{1}H NMR spectra of **NTBT** without and with the addition of DCP in DMSO-d6 (Fig. S11, ESI†). Upon an incremental addition of DCP, the characteristic proton signal at 4.45 ppm for the quaternized N-CH_3 protons was gradually shifted upfield. The shift in the signals was attributed to the reduced positive charge on quaternary nitrogen atom of the indole ring by nucleophilic attack of DCP P=O to the electrophilic centre of the indole spirocarbon. Gradual appearance of the ethyl protons was observed in the region of 1.45 ppm and 3.67 ppm upon addition of DCP.

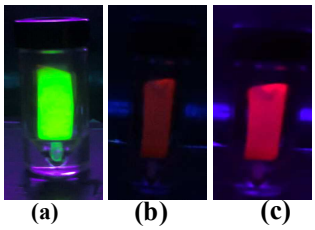


Fig. 2 Display of vapour phase sensing of DCP using a **NTBT** coated paper strip under a UV lamp: (a) only **NTBT** coated filter paper (b) after 15 seconds incubation of DCP (c) after 30 seconds incubation of DCP.

After demonstrating the sensing ability of the chemosensor **NTBT** successfully, we then intended to extend the applicability of this probe for detection of DCP in solution and gaseous state which is based on a portable chemosensor kit. To make the detection experiments operationally simple and practical, Whatman filter paper was used. It was immersed into the DMSO solution of **NTBT** (0.1 mg /mL) then dried in air. The white filter paper changed to a deep green color and after a spray of DMSO solution of DCP, the color immediately turns yellow under ambient light (Fig. S21a, ESI†). As shown, an obvious immediate color change occured under a hand held UV lamp (Fig. S21b, ESI†), accompanied by an enhanced red fluorescence that could be clearly observed by the naked eye.

In gaseous state studies, the **NTBT** containing dry filter papers were kept hanging from the inside wall of a glass chamber so that it

remained above the bottom level. Interestingly, when a few drops of DCP were added into the glass chamber, an immediate 'turn-on' response was observed (Fig. 2). The responsive nature of the **NTBT** soaked filter paper strip towards DCP in the presence of other chlorinated compounds remained unperturbed.

Finally, we attempted to apply probe **NTBT** for the ratiometric fluorescence imaging of DCP in live biological sample for the first time. The intense intracellular green fluorescence of the cells (RAW 264.7 macrophage cells) incubated with probe **NTBT** (0.11 μM) for 20 min demonstrated that probe **NTBT** is cell permeable (Fig. 3c). Interestingly, the fluorescent green cells changed to red on exposure of the cells to 1.1 μM DCP for another 5 min. (Fig. 3g). As expected, distinct changes in ratiometric fluorescence responses generated from the green channel and the red channel in living cells were observed (Fig.3).

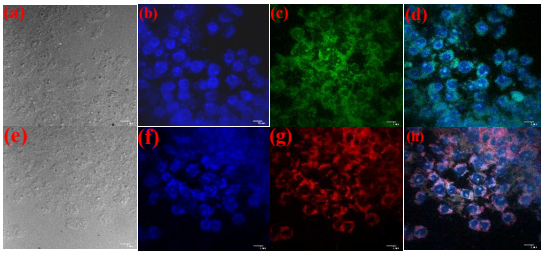


Fig. 3 Confocal fluorescence images of probe in Raw 264.7 cells (40× objective lens): (a) Bright field image of the cells (b) nuclei counter stained with DAPI fluorescent stain (1 mg mL^{-1}) (c) Stained with probe **NTBT** at concentration 1.1 x 10^{-7} M (green channel, λ_{ex}=488nm, λ_{em}= 510 - 560 nm) (d) Overlay image of (b) and (c) in dark field (e) bright field image of RAW cells treated with DCP (1.1 x 10^{-6}) only (f) nuclei of (e) counter stained with DAPI fluorescent stain (1 mg mL^{-1}) (g) Stained with probe **NTBT** at concentration 1.1 x 10^{-7} M(red channel, λ_{ex}=488nm, λ_{em}= 580 - 630 nm) (h) Overlapping image of (f) and (g).

More attractively, the little changes in DCP levels were also clearly observed by the ratiometric fluorescence imaging, implying that our proposed probe **NTBT** possesses high resolution in bioimaging.[22] These results revealed that probe **NTBT** could be used for the ratiometric fluorescence imaging of DCP in living matrices. The cytotoxicity of **NTBT** was examined towards RAW cells by a MTT assay (Fig. S25, ESI†).

In conclusion, we have developed for the first time a new ratiometric fluorogenic and chromogenic probe **NTBT** for nanomolar detection of DCP, based on a napthothiazolium–benzothiazole conjugate. The detecting mechanism is based on tandem phosphorylation via irreversible ring-opening and ring-forming processes. The probe displays clearly DCP-induced changes in the intensity ratio of two well-separated emission peaks of the conjugate with a large red-shifted emission of 135 nm and a drastic color change from green to red. Paper strips of **NTBT** show an instantaneous response upon exposure to DCP, and hence can be utilized for in-field applications. Furthermore, we have demonstrated that the probe is suitable for ratiometric imaging of variations of DCP levels in living cells.

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