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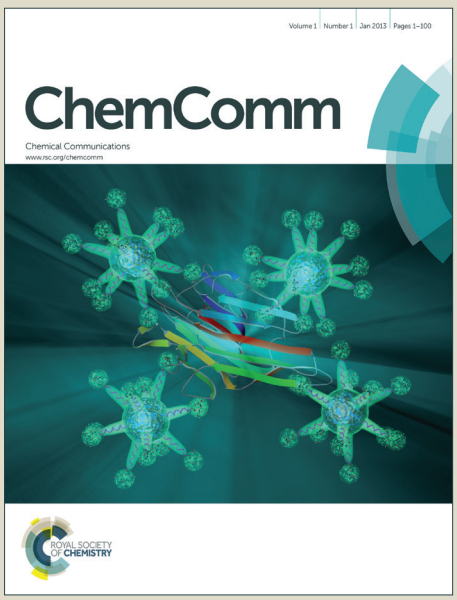

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Intramolecular crossed-benzoin reaction based KCN fluorescent probe in aqueous and biological environments†

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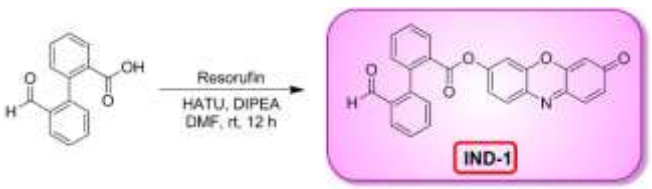
A turn-on fluorescent probe IND-1 was designed for selective cyanide anion sensing in aqueous and biological environments. The probe underwent an intramolecular crossed-benzoin reaction in the presence of KCN to expel the fluorophore resorufin. This probe was sensitive to KCN concentrations as low as 4 nM in aqueous media.

Fluorogenic probes are widely used to detect biologically active molecules, therapeutics, proteins, and ions because they are easy to modify, cheap, and exhibit rapid responses to the analytes.[1] A fluorogenic probe for sensing CN^- is crucial as this ion is extremely poisonous to human health, especially in its strong ionic form, *e.g.* KCN and NaCN. CN^- ions inhibit the cellular respiratory process by disrupting the function of cytochrome c oxidase; this ion forms a stable complex with a heme unit in the active site of cytochrome a_3.[2] In cellular environments, CN^- ions induce an enhancement of the level of intracellular calcium ions, causing cascades of enzymatic reactions that increase the cellular reactive oxygen species (ROS), which eventually damage the human oxidative immune defence system.[3] Nevertheless, cyanide is widely used in mining and other industries for gold and silver extraction, tanning, electroplating, etc. Moreover, cyanide can be used in chemical warfare for mass-destruction in human society.[4] Thus, the rapid, unambiguous, and selective detection of CN^- ions in aqueous environments by employing an eye-catching optical method is crucial. There have been efforts to develop CN^- sensors based on H-bonding motifs,[5] oxazines,[6] cationic borane derivatives,[7] acridinium salts,[8] β-turn motifs,[9] fluorogenic ensembles,[10] and reaction-based chemodosimeters.[11] Most of these systems give a response either through colorimetric or fluorescence changes; a few cases showed both colorimetric and fluorescence changes that could be observed by the naked eye.[10c,11a] However, most of the reported CN^- sensors work with NH_4CN in non-aqueous environments;[8,9,11e,12] there are few reports on KCN or NaCN sensing in aqueous environments.[11a,11c,11g] Additionally, KCN and NaCN are more highly toxic than NH_4CN; thus, it is crucial to devise a fluorescent probe to detect KCN and NaCN in aqueous environments. Herein we report a strategy-based chemodosimeter as a fluorescent probe for KCN sensing in aqueous environments (Scheme 1). The probe, **IND-1**, was synthesized in a single step by esterifying 2'-formyl[1,1'-biphenyl]-2-carboxylic acid with resorufin. The details of the synthetic procedure and spectroscopic evidence are provided in the supplementary information.



Scheme 1 Synthesis of probe **IND-1**.

In the presence of KCN, we presumed a carbanion would be generated at the aldehyde carbonyl of **IND-1** via a benzoin-type reaction. The close proximity of the ester carbonyl would allow intramolecular cross-coupling with the expulsion of free resorufin, which shows strong fluorescence.

To determine the sensing ability of **IND-1**, we examined the UV-absorption changes of **IND-1** in the presence and absence of KCN in PBS buffer. The UV-absorption centred at 565 nm increased 20-fold in the presence of KCN (10 equiv.) with a visible change from pale orange to deep pink, as seen in Fig. S1a (ESI†); a new emission band appeared at 595 nm (Fig. S1b, ESI†) when excited at 565 nm. To determine the sensitivity of **IND-1** toward CN^- ions, we measured the concentration dependent fluorescence changes. In Fig. 1a, the fluorescence intensity at λ_{em} = 595 nm gradually increases with increasing concentrations of CN^- (0–3 equiv.). We determined the lower detection limit to be 4 nM (Fig. 1c), which is much lower than the permissible cyanide concentration (1.9 μM) in drinking water prescribed by the World Health Organization (WHO).[13] To the best of our knowledge, this is the lowest detection limit reported for the sensing of CN^- ions. Interestingly, **IND-1** is capable of detecting CN^- ions in human blood serum using the fluorescence enhancement at λ_{em} = 595 nm at CN^- ion concentrations as low as 50 nM, as shown in Fig. S2 (ESI†). This result implied that **IND-1** could be used to detect CN^- ions in living systems.

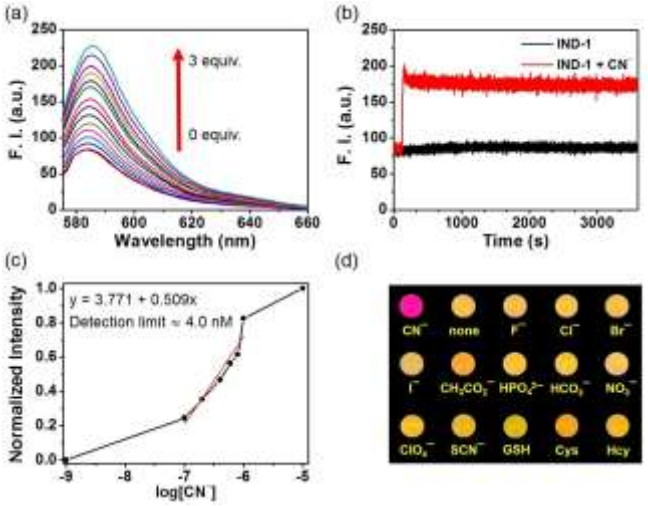


Fig. 1 (a) Fluorescence spectra of **IND-1** (10 μM) with various concentrations of CN^- (0–3 equiv.). (b) Time dependent changes in the fluorescence intensity of **IND-1** (10 μM) with and without 10 equiv. of CN^- in PBS buffer (10 mM, pH 7.4) with 1 % DMSO (λ_{ex} = 565 nm, λ_{em} = 595 nm, excitation and emission slit widths: 1.5 nm). (c) Fluorescence intensity of the probe, normalized for the minimum fluorescence intensity in the absence of CN^- (shown on the graph as 1 nM) and the maximum fluorescence intensity (1 μM CN^-). (d) Color changes of test strips containing **IND-1** (10 μM) treated with various anions (CN^-, none, F^-, Cl^-, Br^-, I^-, $CH_3CO_2^-$, HPO_4^{2-}, HCO_3^-, NO_3^-, ClO_4^-, SCN^-, GSH, Cys, Hcy; 10 equiv.).

To implement sensing in real systems, the detection time for CN^- ions is crucial. Thus, we carried out time-course experiments in the presence of KCN (10 equiv.) in PBS buffer (Fig. 1b). Within 10 s resorufin was fully released from **IND-1**; consequently, the fluorescence signal recovered and reached saturation. This result suggested that **IND-1** is very sensitive and has a fast response to CN^- ions.

In practice, the selectivity of **IND-1** toward a particular analyte in the presence of other interfering anions is very important. We recorded the fluorescence changes of **IND-1** in the presence of the potassium salts of various anions, such as CN^-, F^-, Cl^-, Br^-, I^-, $CH_3CO_2^-$, HPO_4^{2-}, HCO_3^-, NO_3^-, ClO_4^-, and SCN^-, and some neutral species, such as Cys, Hcy, and GSH (Fig. S3, ESI†). **IND-1** was observed to be highly selective for CN^- ions; there was no significant change in the fluorescence signal in the presence of other anions. Fig. 1d depicts the color changes of test strips containing **IND-1** in the presence of various analytes. Interestingly, only CN^- ions caused a color change to pink, whereas the color was unaltered with the other anions. This result suggests that **IND-1** can be used for high throughput screening of KCN in immobilized systems, even in the presence of other ions.

The pH sensitivity of the **IND-1** reaction with KCN was determined by monitoring the changes in the fluorescence intensity at 595 nm at various pH values (4–9). There was no change in the fluorescence intensity over this pH range (Fig. S4, ESI†), indicating that **IND-1** can be used to sense CN^- ions over a wide range of pH values.

Curiosity drove us to determine the mechanism of action of **IND-1** toward CN^- ions. Thus, we carried out a ^{1}H-NMR titration experiment in the presence of KCN at variable time intervals. Within 5 min, the aldehyde proton peak in **IND-1** at 9.78 ppm (★) was observed to disappear completely (Fig. 2) with concomitant appearance of a new set of peaks at 7.52–8.22 ppm. To identify the new set of proton peaks, we recoded the ^{1}H-NMR spectra of resorufin and 9,10-phenanthrenequinone. The proton signals and integration ratios for resorufin and 9,10-phenanthrenequinone were found to exactly match those observed in the ^{1}H-NMR spectra of KCN-treated **IND-1** (Fig. 2).

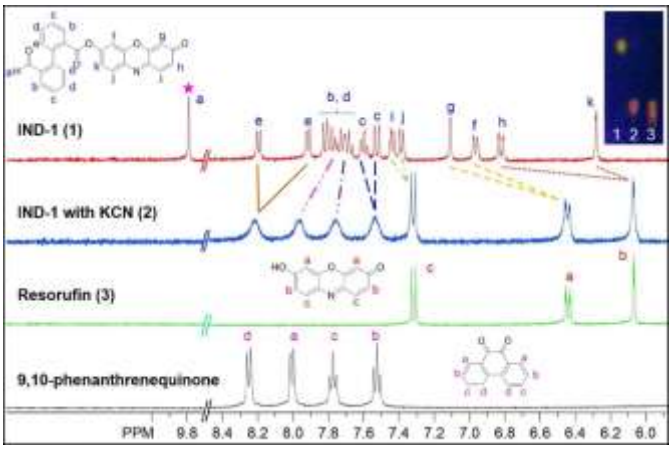


Fig. 2 ^{1}H-NMR spectra (400 MHz, DMSO-d_6 with 10% D_2O) of **IND-1** (red), **IND-1** with CN^- (blue), resorufin (green), and 9,10-phenanthrenequinone (black). Inset: photograph of **IND-1** (1), **IND-1** (10 μM) with 10 equiv. of CN^- (2), and resorufin (3) taken under a UV-lamp at 365 nm.

The mass spectrum data of KCN-treated **IND-1** (Fig. S10, ESI†) also showed revealed that 9,10-phenanthrenequinone was formed. Moreover the TLC profile (Fig. 2, inset) of KCN treated **IND-1** was exactly matched with resorufin and 9,10-phenanthrenequinone respectively. These experimental findings strongly supported our speculation on KCN catalyzed intramolecular crossed-benzoin reaction (Scheme 2) that produces the discrete new molecule, 9,10-phenanthrenequinone.

Scheme 2 KCN catalyzed intramolecular crossed-benzoin reaction of **IND-1**.

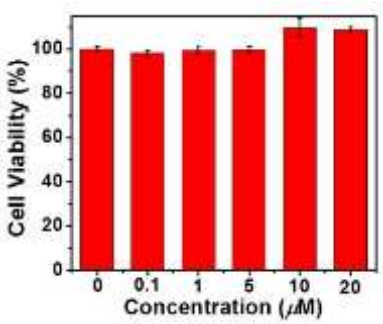


Fig. 3 Cell viability assay of **IND-1** on HeLa cell lines.**IND-1** was incubated with the cells for 24 h, and the cell viability observed via MTT assay.

We then evaluated biocompatibility of **IND-1** by MTT assay. Data in Fig. 3 revealed that until 20 μM of **IND-1** cells remain healthy without change of cell viability. It indicates **IND-1** can be used in cellular level to assess the presence of CN^- ions.

Finally, we evaluated whether **IND-1** can show the KCN catalyzed intramolcular crossed-benzoin reaction to give cell image based on cellular CN^- ions. Fig. 4a indicates that fluorescence intensity gradually increases with increasing conc. of KCN. The cell images in Fig. 4a and S5, implied that KCN in cells can be detected using this **IND-1** irrespective of various cell lines (HeLa and A549). In addition, we carried out co-localization experiment to know the distribution of KCN in cells and justify whether **IND-1** is capable to note the KCN throughout the cells. The Fig. 4b concluded that fluorescent images of **IND-1** co-localized with Mito-tracker and ER-tracker as well.

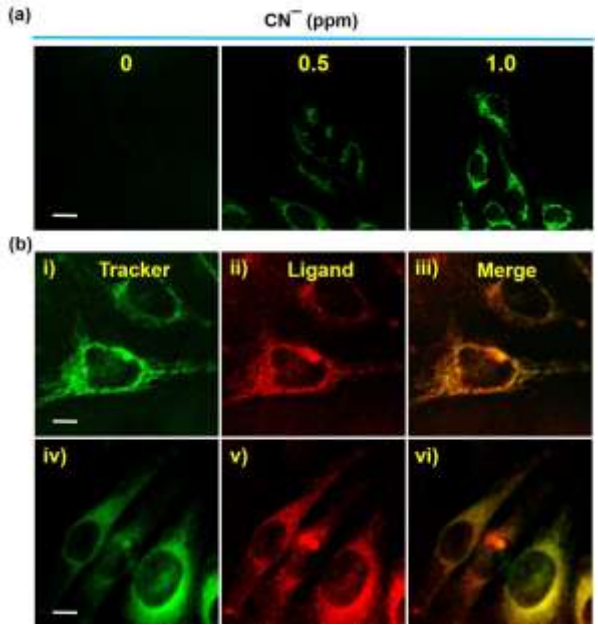


Fig. 4 (a) Confocal laser fluorescence microscopic images of HeLa cells treated with **IND-1** (2 μM). The cells were pre-incubated with media containing CN^- of various concentrations (0, 0.5 and 1.0 ppm) for 30 min at 37 ℃. Cell images were obtained using excitation wavelengths of 543 nm, and emission wavelengths of 570-630 nm, green signal, respectively. (b) Confocal microscopic images of co-localized experiment in HeLa cells. (ii) and (v) Fluorescence images of HeLa cells contained with **IND-1** (5 μM) for 20 min. The cells were pre-incubated with media containing CN^- (0.5 ppm) of 30 min. (i) Mito tracker green FM (0.5 μM) for 10 min. (iii) Overlay of the merged images of (i) and (ii). (iv) ER-tracker green (0.5 μM) for 10 min. (vi) Overlay of the merged images of (iv) and (v). Images of the cells were obtained using excitation wavelengths of 514 nm and 543 nm, and a band path (520-550 nm, green signal) and (570-630 nm, red signal), emission filters, respectively. scale bar: 10 μm

Further, Mito-tracker, ER-tracker, and **IND-1** were subjected to fluorescence profile studies through the transverse section of the HeLa cells. The fluorescence profile of the HeLa cells treated with **IND-1** exactly matched the profiles of the HeLa cells labelled with Mito-tracker and ER-tracker (Fig. S6, ESI†). These results led us to conclude that **IND-1** is an efficient probe to track KCN throughout cells, including sub-cellular organelles.

In conclusion, we developed for the first time a molecular probe, **IND-1**, for sensing KCN in aqueous environments based on an intramolecular crossed-benzoin reaction. The synthesis of this turn-on fluorescent chemodosimeter probe was very simple, and **IND-1** was shown to be an efficient probe with a fast response in two modes *i.e.* as a chromogenic and fluorogenic sensor for KCN in aqueous and biological environments**. IND-1** displayed sensitivity toward concentrations of KCN as low as 4 nM, which is remarkably lower than any previously reported system. Further**, IND-1** is capable of detecting KCN at a concentration of 50 nM in blood serum by amplifying the emission signal at 595 nm. Finally, cellular imaging and co-localization experiments strongly suggested that **IND-1** could be used to track KCN in cellular environments.

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Notes and references

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† Electronic Supplementary Information (ESI) available: Synthesis procedure, NMR, MS, fluorescence, and cell imaging data. See DOI: 10.1039/c000000x/

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