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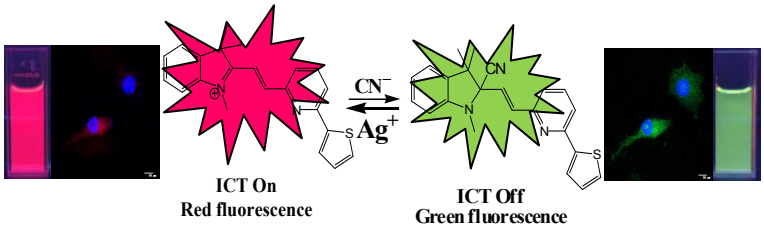
Ajit Kumar Mahapatra,* Kalipada Maiti, Rajkishor Maji, Saikat Kumar Manna, Sanchita Mondal, Syed Samim Ali and Srimanta Manna

An indole conjugated thiophene-pyridyl moiety, **ITP** has been synthesized and characterized for selective detection of cyanide with low detection limit.



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ARTICLE TYPE

Ratiometric fluorescent and chromogenic chemodosimeter for cyanide detection in water and its application for bioimaging

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An indole conjugated thiophene-pyridyl **ITP** for cyanide sensor has been synthesized and characterized by NMR and mass spectroscopy. The selectivity of **ITP** has been explored in aqueous solution, resulting ratiometric fluorescence response toward CN^- among 11 different anions studied. The complexation of **ITP-CN** has been addressed by HRMS, ^1H NMR, and UV−vis spectra. **ITP** displays substantial dual changes in both ratiometric emission and absorption spectra exclusively for CN^- in aqueous solution. Due to the nucleophilic attack of CN^- towards the indolium group of **ITP,** by which ratiometric fluorescence change and consequently a large emission shift has been achieved. DFT/TDDFT calculations were performed in order to demonstrate the electronic properties of **ITP** and their **ITP-CN** adduct. The resultant **ITP-CN** adduct, has been used as a secondary sensing chemoensemble for the detection of cyanophilic metal ions containing molecules by removing CN^- from **ITP-CN** and regenerating **ITP** by switch-on red fluorescence. For the practical application of the sensor, the test strips based on **ITP** were made-up, which could act as suitable and proficient CN^- test kits and cell studies.

Introduction

Fluorescence chemosensor are selectively recognizing the toxic and lethal anionic species and hence receiving considerable attention in chemistry, biology, medicine and in relation to environmental issues.[1] Among them cyanide is a first-acting, potentially deadly chemical that prevents the cells of the body from using oxygen properly. Not only it can affect many functions in the human body, including the vascular, visual, central nervous system, cardiac, endocrine, and metabolic systems[2]. Cyanide exists in various forms including gaseous hydrogen cyanide (HCN), water-soluble potassium or sodium cyanide salts, and in some cyanogens. Metal extraction in mining, electroplating in jewellery production, photography, plastics and rubber manufacturing, hair removal from hides, and rodent pesticide and fumigants have all been implicated in cyanide poisonings.[3] A significant proportion of fatalities among fire victims is due to cyanide poisonings, as blood cyanide concentrations reach a level of 23–26 μM.[4] Especially, KCN is a potent poison, inhibiting cytochrome oxidase and thereby the cells' respiration by forming a permanent bind with the iron atom in heme of cytochrome.[5]

Taking these considerations into account, several receptors as optical sensors have been proposed for cyanide ion detection.[6] However; many of these sensors rely on a hydrogen-bonding motif in organic solvent and have generally displayed moderate selectivity over other anions.[7]

To overcome this problem there are design and synthesis of some reaction based cyanide sensors developed recently; these are oxazines,[8] cationic borane derivatives,[9].acridinium salts,[10] and b-turn motif.[11] But the fact is that many of them display either color changes or fluorescence changes, individually, but there are some limited examples where the receptors showing simultaneous changes in both absorption and emission spectra.[7-13]. Nevertheless, a few chemical sensors[14], that are operating in water and show both the colorimetric and fluorescence changes upon the complexation of cyanide anions.[15] Moreover, those dual spectral-changed based sensors are showing fluorescence quenching (On-Off) in their corresponding **CN**-adduct compound.[11-14] So it is a challenge to the organic chemist to design and synthesis of a fluorescence chemosensors for cyanide which can shows both color and fluorescence changes in aqueous medium on a ratiometric manner.

Most of the cyanide sensors reported, functioning as a fluorescence quenching or enhancement. As the change in fluorescence intensity is the only detection signal, factors such as instrumental efficiency, environmental conditions, and the probe concentration can interfere with the signal output. Ratiometric[16] sensor have better utility than fluorescence based chemosensor (On or Off) as it measured the fluorescence intensity at two different wavelengths, which increase the dynamic range and provide a build-in correction for environmental and concentration effects. In general, ratiometric sensors can be functioned by the following two mechanisms: fluorescence resonance energy

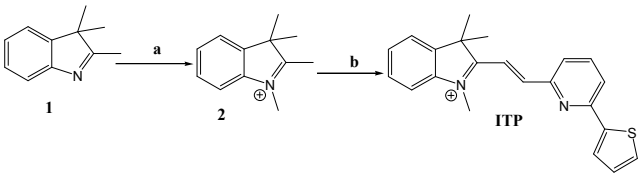
transfer (FRET) [17] and intramolecular charge transfer(ICT).[18] By contrast ICT based sensor are structurally very simple and easy to make. Here, we report a new indole conjugated thiophene-pyridyl (**ITP**) probe which shows a ratiometric fluorescence changes with CN^- by ICT blocking in aqueous solution.

The choice of indole moiety conjugated to thiophene-pyridyl moiety as a chromogenic sensory molecule was mainly based on the fact that the conjugated indole skeleton could function as a color-reporting group. At the same time conjugated indole based chemosensor showed their favorable photo physical properties including emission wavelength beyond 600 nm and a relatively large stoke shift.[19] So, conjugation of indole with thiophene-pyridyl moiety as seen in Scheme 1 might modulate the internal charge transfer (ICT) state and give rise to large dual color and fluorescence changes in the presence of CN^-.

ITP shows intense red emission due to the extended π-conjugation and a strong ICT from pyridyl moiety through ethylenic group towards the indolyl moiety. Hence, it is expected that cyanide attack towards the indolium moiety not only interrupt the π-conjugation but also blocking the ICT process. This is why a hypsochromic shift occur both in absorbance and emission spectra of **ITP-CN** adduct. However, from our best knowledge only a few report on conjugated moiety based ratiometric fluorescence version of cyanide sensor have been exploited before[20].

Results and discussion

Probe **ITP** was synthesized conveniently *via* a simple condensation between 6-(2-thienyl)-2-pyridinecarboxaldehyde and N,2,3,3-tetramethylindolium cation in ethanol. Structure of the compounds was identified by ^1H NMR, ^{13}C NMR, and HRMS spectroscopy Fig. S1, S2, S3, †).



Scheme 1 Scheme for the synthesis of **ITP**: (a) CH_3I, $CHCl_3$, r.t; (b) 6-(2-thienyl)-2-pyridinecarboxaldehyde, EtOH, reflux.

The sensitivity of **ITP** toward different anions and their preferential selectivity toward CN^- over the other anions has been studied by fluorescence and absorption titrations. The ion recognition of **ITP** has been studied by exciting the solutions (basic buffer at pH 9.3) at 411 nm and measuring its emission spectra from 450 to 800 nm. In order to make sure CN^- to nucleophilic addition of **ITP** in aqueous basic buffer solution at pH 9.3, fluorescence titrations were carried out in 50 mM aqueous HEPES buffer taken with DMSO-H_2O (5:95, v/v) to give an effective buffer concentration of 10 mM. Among 11 anions (n-Bu_4N^+ salts of F^-, Cl^-, Br^-, I^-, SO_4^{2-}, HSO_3^-, AcO^-, HS^-, $H_2PO_4^-$, and K^+ salts of, NO_2^- and CN^-) only CN^- shows remarkable color change both in naked eye and fluorescence.

Titration of **ITP** with CN^- showed a blue emission shift 115 nm in the λ_{em} maximum of **ITP**. Titration of this by CN^- results in a gradual quenching of the fluorescence emission at 619 nm band and a new emission band appears at 504 nm as a function of the increased CN^- concentration in a ratiometric manner (Fig. 1). A clear isoemissive point at 556 nm also indicating the formation of **ITP-CN** adduct.

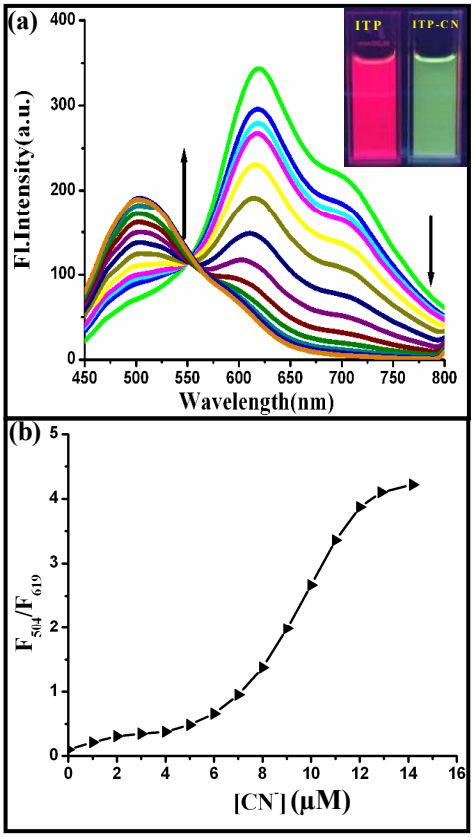


Fig. 1 Fluorescence spectral changes of **ITP** (1.0 x10^{-6} M) in DMSO-H_2O (5:95 V/V; pH 9.3) upon addition of CN^- (c = 4.0 x10^{-6} M). The inset shows fluorescent color change of **ITP** in presence of CN^-. (b) Change in ratio of fluorescence intensity at 504 nm & 619 nm of **ITP** as a function of CN^- concentration.

We carried out time-dependent cyanide adduct experiment on ITP (1.0 μM) in the presence of varying amounts of cyanide anion (0.5, 2.0, 5.0, and 10.0 μM) and observed a significant increase in the fluorescence intensity over a period of 90, 65, 50, and 40 seconds respectively (Fig. S14, †). We also calculated rate constants for the above-described time-dependent measurements by plotting intensity versus time (Fig. S15, †). The plot clearly demonstrates that the reaction follows pseudo first-order kinetics, and the rate constants for each concentration of CN^- (0.5, 2.0, 5.0, and 10.0 μM)) were found to be 0.026, 0.0258, 0.0261, and 0.026 s^{-1} respectively, which are almost the same irrespective of cyanide concentrations as it should be. The minimum concentration of CN^- that can be detected by **ITP** using fluorescence titration has been found to be 1.5 μM **(**Fig. S6, †**)** signifying that it operating well below the WHO cyanide standard in drinking water (1.9 μM).

In order to check whether **ITP** is sensitive to only CN^- or even to the other ions, competitive anion fluorescence titrations were carried out in the same medium with 10 different anions, viz. F^-, Cl^-, Br^-, I^-, SO_4^{2-}, HSO_3^-, AcO^-, HS^-, $H_2PO_4^-$, and NO_2^-, and no significant fluorescence enhancement was found in the presence of these ions (Fig. 2a).

During the titration, the **ITP**:A^{n-} ratio was kept at 1 : 10 and the resultant solution was titrated against varying concentrations of CN^- for each A^{n-}. It was seen that only HS^- shows a little interference while the other anions did not lead to any significant ratiometric fluorescence change, and the fluorescence emission spectra of **ITP** remain unaltered (Fig. 2b). Even the competitive titrations carried out by keeping **ITP**: CN^- ratio of 1: 1 and varying the concentration of the other A^{n-} showed similar results.

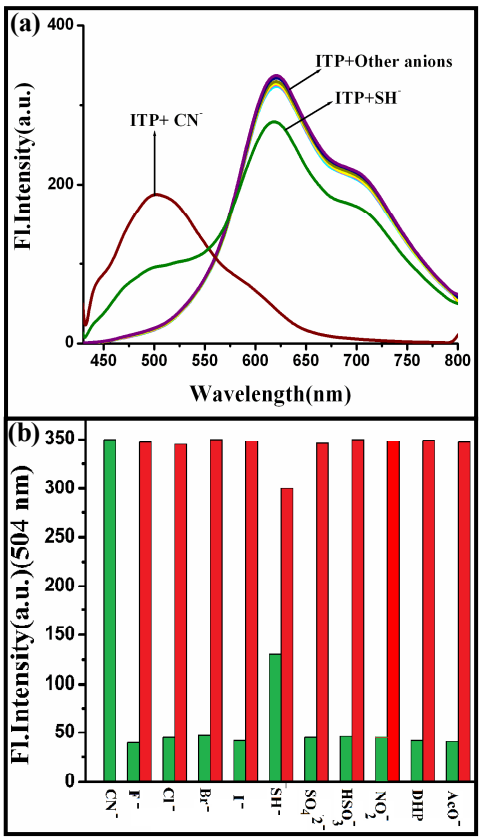


Fig. 2 (a) Fluorescence spectral change of **ITP** (1.0 x10^{-6} M) with the addition of different anions (4.0 x10^{-6} M) in DMSO-H_2O (5:95 V/V; pH 9.3). (b) Competitive graph; green bar: **ITP** + anions, red bar: **ITP** + anions + CN^-.

In order to support the results obtained from the fluorescence studies, absorption titrations were carried out. The absorbance of **ITP** exhibited three characteristic bands at 246, 305, and 411nm in buffer solution. While the absorbance of the 305 and 411nm bands decreases that of the 246 nm band increases (Fig. 3a) on gradual addition of KCN solution up to 7 equiv. for saturation; eventually, an obvious color changes dark brown to colorless was clearly observed, indicating that ICT is turned off due to the nucleophilic attack by CN^- at the indolyl cation of **ITP**. Thus the spectral changes and the isosbestic point observed at 260 nm clearly suggest the formation of a new species by nucleophilic addition reaction between **ITP** and CN^- while, the other anions exhibit no significant change in the absorption spectra (Fig.S7, †).

The CN^- sensing property of **ITP** has been further supported by observing the fluorescent color change visually in the presence of different anions under an incident light of 254 nm, and found green fluorescence only in case of CN^-, while the other ions do not show such emission (Fig. S13, †). Further, this has been carried out in the presence of other anions added to an initial solution possessing a **ITP-CN** adduct and found no changes in the green fluorescence in case of all the anions, suggesting that the CN^- sensing by **ITP** can be monitored even in presence of all the other more or less nucleophilic anions . The formation of 1:1 stoichiometric reaction product is confirmed by HRMS analysis where an CH_3CN solution of **ITP** with CN^- showed a peak at m/z 394.1543 corresponding to **[ITP+CN+Na]**$^+$, clearly observed (Fig. S4, †).

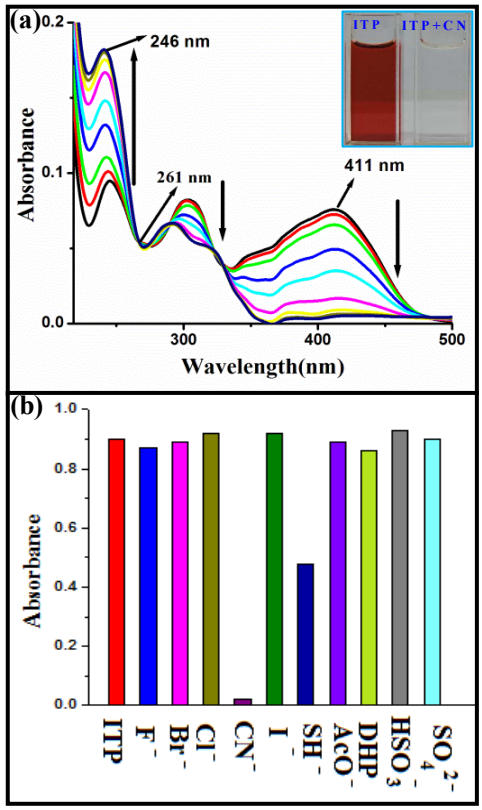


Fig. 3 (a) UV-vis spectral changes of **ITP** (1.0 x10^{-6} M) in DMSO-H_2O (5:95 V/V; pH 9.3) upon addition of CN^- (4.0 x10^{-6} M).The inset shows naked eye color change of **ITP** (1.0 x10^{-4} M) and addition of CN^- (1.0 x10^{-3} M) to the **ITP** in DMSO-H_2O (5:95 V/V; pH 9.3) . (b) Change in absorption intensity of **ITP** (1.0 x10^{-6} M) (at 411 nm) during titration with different anions (4.0 x10^{-6} M).

In addition we also examine the ^{1}HNMR titration of **ITP** with CN^- in d_6-DMSO. After the addition of 2equiv. of CN^- (KCN) a significant proton shifting occurs. However, the product of **ITP-**

CN leads to a upfield shift of the olefinic proton , indicating that the electron withdrawing effect by indole quaternary N atom decreases. The vinyl protons at δ 8.70 (Hi) and δ 8.45 (Hj) were upfield shifted to δ 8.28 and δ 8.23 respectively (Fig. 4). The N-CH_3 protons of **ITP** form are also magnetically deshielded due to the decreased electron density of the indole ring by quaternization of the nitrogen atom and upfield shifted after the addition of CN^-(from δ 4.7 to δ 2.9).

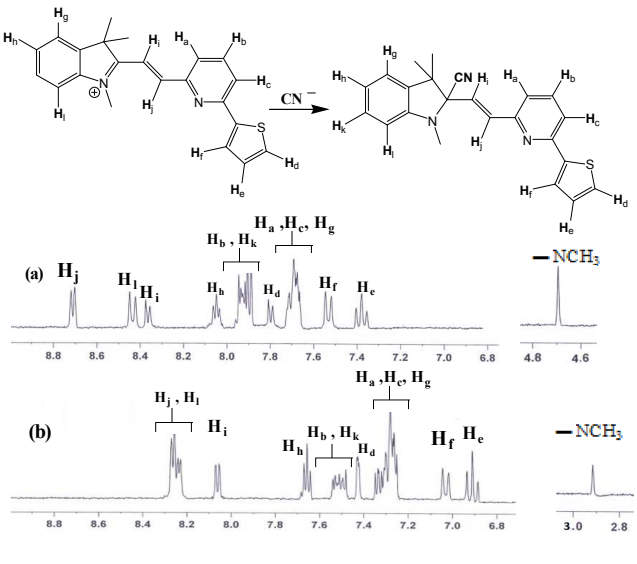


Fig. 4 ^{1}H NMR chart (300 MHz, DMSO-d_6 , 0.5 ml) of **ITP** (10 mg) measured (a) without CN^- and (b) with addition of CN^- (2.5 equiv.)

To understand the absorption and fluorescence phenomenon of **ITP** in presence of CN^-, density functional theory (DFT) calculations were performed with a suite of Gaussian 03 package[21]. Change in fluorescence spectra of **ITP** with the addition of CN^- can be explained by time-dependent DFT (TDDFT) as well.

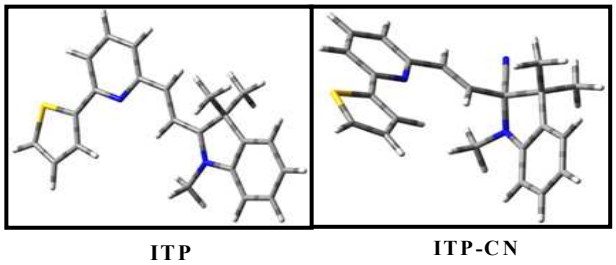


Fig. 5 The energy optimized structures of **ITP** and **ITP-CN.**

The calculated HOMO-LUMO energy gap of **ITP** and **ITP-CN** are 2.30 and 3.82 eV respectively (Table S1, †). For **ITP** alone, there are two peaks in its absorption spectra at 305 nm and 411 nm. These two bands arises due to the electronic transition of HOMO→LUMO+1(3.93 eV/315 nm) and HOMO-1→LUMO (3.06eV/405 nm) respectively. HOMO-1→LUMO transition is the most fundamental transition as it shows high percent of transition(64%) and having a greater value of oscillating strength (0.71) (Table S1, †**).** In case of **ITP-CN** adduct HOMO-1→LUMO+1 transition is the most fundamental. The electron densities in the LUMO of **ITP** are distributed to the indolyl moiety through the thiophene-pyridyl (**TP**) moiety while, in case of **ITP-CN** the electron densities of LUMO, LUMO+1 and LUMO+2 only reside on thiophene-pyridyl (**TP**) moiety.

There is a considerable difference in the energy minimization structure in **ITP** and **ITP-CN** adduct, which can shed light on the change in absorption spectra and the corresponding change in color. Energy minimization structure of **ITP** clearly shows the total planarity of the compound. While the energy minimization structure of **ITP-CN** shows that after CN^- attack, an indolyl group adopted tilted geometry and becomes perpendicular to the pyridyl group (Fig. 5). This structural difference gives rise to difference in π-conjugation between **ITP** and **ITP-CN**, and hence the ICT blocking. So, the shifting of fluorescence towards the blue region in the **ITP-CN** adduct is mainly due to ICT blocking.

Owing to the well-known strong affinity of cyanide toward the Ag^+, the weak fluorescent cyanide-adduct [**ITP-CN**] has been studied for their secondary sensing property toward cyanophilic cations.

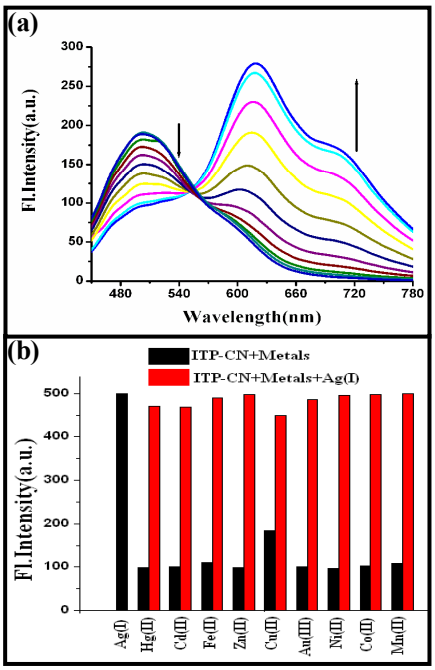
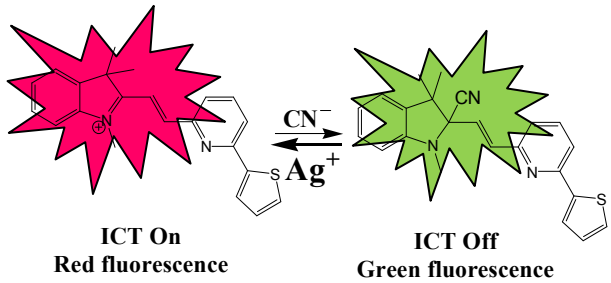


Fig. 6 (a) Fluorescence spectral change of **ITP-CN** (c= 1× 10^{-6} M) when treated with Ag^+(c= 2×10^{-5} M). (b) Change in Fluorescence response of **ITP-CN** (c= 1×10^{-6} M) to 2 eqv. addition of Ag^+ (c= 1×10^{-5} M) and 7 eqv. of other metal ions (c= 1×10^{-5} M) [the black bar portion] and to the mixture of 7 eqv. of other metal ions with 2 eqv. addition of Ag^+ [the red bar portion].

To our delight, the reversible process did happen and can be rationalized by aza-SN$^{2'}$ displacement pathway triggered by Ag^+. During the titration of [**ITP-CN**] with cyanophilic cations, only Ag^+ caused the remarkable increase of the fluorescence emission band at 619 nm and simultaneously the emission band observed at 504 nm is quenched (Fig. 6), which provides an alternative new approach for the undeveloped fluorescent sensing of silver

ions. This is exactly reverse to what happens when **ITP** is titrated with CN^-, indicating the removal of CN^- by Ag^+ and thereby releasing the free **ITP**. Inevitably this is true with Cu^{2+}, Au^{3+}, and Au^+ also increased the emission intensity to a small extent and which show saturation at much higher equivalents, respectively. Thus the [**ITP-CN**] complex acts as a secondary recognition ensemble toward Ag^+. Interaction of CN^- with Ag^+ and the consequent release of **ITP** were further supported by UV−visible absorption spectroscopy carried out with [**ITP-CN**]. Absorption spectrum obtained after addition of Ag^+ to [**ITP-CN**] is similar to the free **ITP** showing bands at 411nm, 305 nm and 246 nm, suggesting the release of **ITP** from the adduct by Ag^+ (Fig. S8, †).



Scheme 2 Schematic presentation of cyanide binding mode with **ITP** and regeneration of **ITP** from **ITP-CN** adduct by Ag^+.

From the naked eye detection it is found that **ITP** shows characteristic color changes in presence of cyanide in solution.

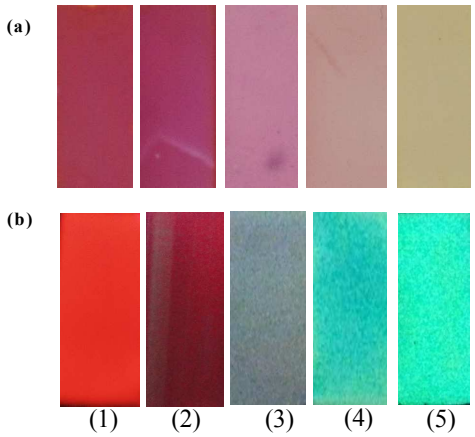


Fig. 7 (a) Naked eye in room light and (b) Fluorescence color changes visualized on TLC plate strips of (1) **ITP** (c = 1.0× 10^{-2} M) and during addition of CN^- at (2) 1.0×10^{-5} M; (3) 1.0×10^{-4} M; (4) 1.0 ×10^{-3} M (5) 1.0 × 10^{-2} M in DMSO/H_2O = 5:95(v/v).

In order to realize that if it will be visualize on TLC plate , test strips were prepared by immersing TLC plates into a water solution of **ITP** (c = 1.0× 10^{-2} M) and then drying them in air. The test strips containing **ITP** were immersed in aqueous solution having different cyanide concentrations to sense CN^- and other anions. When CN^- ion concentration was increased, color of the test strips changes from deep brown to colorless (Fig. 7). And potentially competitive ions did not influence in the detection of CN^- by the test strips. Similarly fluorescent color changes occurs from red to green in aqueous solutions having different cyanide concentrations in the test papers provided the practical means to inspect cyanide anion concentrations in the wilderness. Therefore, the test strips could conveniently detect CN^- in solutions. The above result suggest that this type of solid system protocol may be used to perform as a sensitive and practical ''dip-in'' naked eye cyanide sensors in the near future.

To demonstrate the practical application of the probe (**ITP)** to detect even a minute amount of CN^-, we carried out experiments in living cells. In *vitro* studies established that the newly synthesized **ITP** probe can detect CN^- with excellent selectivity even up to 50μM. Hence, to assess the usefulness of **ITP** as a probe for *in vitro* detection of CN^- by confocal microscopy, RAW cells were used to detect CN^- ions in live cells. We performed MTT assay (Fig. S12, †), which is based on mitochondrial dehydrogenase activity of viable cells to study cytotoxicity of above mentioned compounds at varying concentrations mentioned in experimental method section (†). Fig. 8 shows that probe **ITP** did not exert any significant effect on cell viability; however the CN^- ions had dose dependent adverse effect when cells were treated with varying concentrations of CN^-. The CN-probe complex also had significant adverse effect on cell viability beyond 75μM.

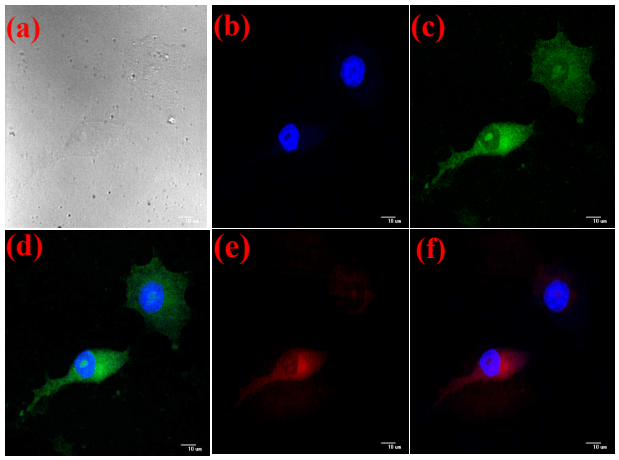


Fig. 8 Confocal fluorescence images of probe in Raw 264.7 cells (40× objective lens) (a) Bright field image of the cells (b) Only KCN at 2.0 x 10^{-5}M concentration and nuclei counterstained with DAPI (1μg/mL) (c) Stained with probe **ITP** at concentration 1.1 x 10^{-5} M (green channel, λ_{ex}=488nm, λ_{em}= 510 - 560 nm) (d) Overlay image in dark field (e) Cells treated with KCN, probe and Ag^+ (c= 1.0 x 10^{-5} M) sequentially when green color goes off and red fluorescence of the probe comes back (red channel, λ_{ex}=488nm, λ_{em}= 580 - 630 nm) (f) Overlay image in dark field when cell treated with KCN, probe and Ag^+.

Exposure of HCT cells to probe **ITP-CN** complex resulted in a decline in cell viability above 20μM concentration. The effect was more pronounced in higher concentration and showed an adverse cytotoxic effect in a dose-dependent manner. [22] The viability of HCT cells was not influenced by the solvent (DMSO) (Fig. S12, †) leading to the conclusion that the observed cytotoxic

effect could be attributed to probe **ITP-CN** complex. The results obtained in the *in vitro* cytotoxic assay suggested that, in order to pursue confocal imaging studies of probe **ITP -CN** complex in live cells, it would be careful to choose a working concentration of 10-20 μM for probe compound. Hence, to assess the effectiveness of compound **ITP** as a probe for intracellular detection of CN^- by confocal microscopy, RAW cells were treated with 20 μM **CN^-** followed by 10 μM probe solution to promote formation of probe **ITP-CN.**

Confocal microscopic studies revealed a lack of fluorescence for RAW cells when treated with CN^- alone (Fig. 8b) and became red fluorescent when treated with **ITP** alone (Fig.S11, †). Upon incubation with CN^- followed by **ITP** a Green fluorescence was observed inside RAW cells, which indicated the formation of **ITP-CN** complex, as observed earlier in solution studies *in vitro* (Fig. 8c). Further, an intense green fluorescence was noticeable in the perinuclear region of RAW cells (Fig. 8d) and again red fluorescence reappeared on treatment with Ag^+ solution (Fig. 8e). The confocal microscopic analysis strongly suggested that probe **ITP** could readily cross the membrane barrier of the RAW cells, and rapidly sense intracellular CN^- in very low concentration. It is significant to mention here that bright field images of treated cells did not reveal any gross morphological changes, which suggested that RAW cells were viable. These findings open up the avenue for future *in vivo* biomedical applications of this sensor.

In conclusion, we have synthesized an indole conjugated thiophene pyridyl compound (**ITP**), which conveniently sensing CN^- ion over other anions. Due to the high nucleophilicity of CN^- , it selectively reacts with the indolium group, than the corresponding other anions. **ITP** shows ratiometric fluorescence changes with CN^- addition and has a large emission shift of about 115 nm. High selectivity and fluorescence behavior of **ITP** was elucidated using DFT/TDDFT calculation. Detection limit of **ITP** is about 1.5 μM, which is lower than the maximum permissible level according to WHO. In addition test strips based on **ITP** were carried out, which also exhibits a good selectivity of CN^- in water. The sensitivity of **ITP** can monitor CN^- in live RAW cell by ratiometric fluorescence imaging.

Experimental

Synthesis

Compound **ITP:** 2,3,3-Trimethyl-3H-indole (**1**) was dissolved in dry $CHCl_3$. Drop wise methyl iodide was added into it and mixture was stirred at room temperature overnight when pale pink precipitate appeared. The cationic salt precipitate was filtered, washed with $CHCl_3$ for several times and collected. 6-(2-thienyl)-2-pyridinecarboxaldehyde (1.05 mmol, 100 mg) and N,2,3,3-tetramethylindolium (**2**) cationic salt (1.05 mmol, 184 mg) were refluxed in 10 ml ethanol solution for 5 h. After reflux the mixture was stirred at room temperature for 1 hr. The solvent was evaporated in vacuum. The red residue was recrystallized by acetic ether/hexane to get the pure product as red crystalline solid (290 mg, 80%). Mp above 250°C. **^{1}H-NMR** ($CDCl_3$, 400 MHz): δ (ppm) 8.61(d, 1H, J=16.0 Hz), 8.28 (d, 1H, J=7.64 Hz), 8.17 (d, 1H, J=16.0 Hz), 7.76 (1H, t, J=8.40 Hz), 7.67 (d, 2H, J=8.30 Hz), 7.64 (d, 1H, J=3.08), 7.62 (m, 3H), 7.45 (d, 1H, J=4.6 Hz), 7.17 (t, 1H, J= 7.20 Hz), 4.51(s, 3H), 1.91 (s, 6H). **Anal. Calcd.** C 76.52, H 6.08, N 8.11, S 9.27; found: C 76.53, H 5.97, N 8.12, S 9.25; **MS (ESI MS)**: (m/z, %): **345.2457**[(**ITP^+**), 100 %]; Calculated for $C_{22}H_{21}N_2S$: **345.4884.** **^{13}C-NMR** (DMSO-d6, 75 MHz): δ(ppm) 181.68, 152.56, 150.87,149.38, 143.80,143.52, 141.82, 138.80, 130.03, 129.57, 129.13, 128.73, 126.88, 126.73, 121.80, 115.75, 115.69, 52.60, 34.52, 24.94.

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Notes and References

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