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# ARTICLE TYPE





## Benzopyrylium-phenothiazine-conjugate of flavylium derivative as fluorescent chemosensor for cyanide in aqueous media and its bioimaging

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A new benzopyrylium-phenothizine-conjugate (**BP**) of flavylium derivative was synthesized and characterized. The probe **BP** was shown to be selective and sensitive for $CN^-$ among the 17 anions and biothiols studied in HEPES buffer medium by fluorescence, absorption, and visual color change. The colorimetric and fluorescent response of the probe **BP** to cyanide ion is due to the Michael addition of cyanide to the activated Michael receptor of the probe which blocks an intramolecular charge transfer process. The probe displays a fast response to cyanide ion at room temperature, and a maximal fluorescent signal is achieved in the presence of only 2 equivalents of cyanide ion. Moreover, the probe **BP** could be used as a practical, visible colorimetric test strip for $CN^-$ in aqueous environment. TDDFT calculations were performed in order to demonstrate the electronic properties of the probe and its cyanide product. Density functional reactivity theory (DFRT) calculation also exhibit for the characterisation of the most electrophilic and nucleophilic centres of the molecule. The probe could be applied for imaging the cyanide in live cells by fluorescence imaging.

### Introduction

Cyanide is well known as one of the extremely toxic rapidly acting and fatal poisons[1] due to its tendency to bind to the heme iron in cytochrome c oxidase,[2] and affects the oxygen supply to the cell which resulted to cellular asphyxiation.[3a-c] However, even very small amounts of the cyanide anion are extremely toxic to living creatures. Cyanide has also been used as a chemical warfare reagent, and even as terror material.[3d] Cyanide is a potent inhibitor of some metalloenzymes and non-metallo-enzymes. This leads to diseases of the vascular, cardiac, visual, endocrine, central nervous and metabolic systems.[4] Despite such a detrimental effect on human health, cyanide is a basic component in the preparation of a wide variety of products[5] ranging from plastic, fibers, gold and silver extraction, tanning, and metallurgy, dyes, chelating agents for water treatment, to pharmaceuticals.[6-7] Cyanide is also released from biological processes of bacteria, fungi and algae even from daily life of human and their activity such as cigarette smoking.[8-9] The seeds of several fruits including apricots, apples and peaches contain substantial amounts of biochemicals that release cyanide when metabolized. Accumulation of cyanide in humans could also happen through consumption of such foods and plants.[10] Thus, the development of cyanide-selective colorimetric or fluorescent probes is in high demand.

A variety of cyanide-selective colorimetric or fluorescent chemosensor probes have been developed over the past ten years by making use of the coordination ability and the nucleophilic reactivity of cyanide.[11] There are two kinds of chemosensors, H-bonding / co-ordination-based sensors and reaction-based sensors, which differ in their signal output and time of response types. Co-ordination-based sensors appear to be particularly attractive due to their fast, convenient and real time monitoring through the formation of metal complexes and metal displacement method.[12] However, the change of signal intensity is depend on several factors such as solvent system, environmental conditions and the sensor concentration can interfere with the signal output. A reaction-based method can overcome the drawbacks of coordination-based sensors since it enables the measurement of emission intensities of new compound generated from irreversible reaction with analytes, thus providing a built-in correction for environmental effects and increasing the dynamic range of absorption/emission measurements.[13]

In recent years, some reaction-based chemosensor probes for cyanide have been reported. For example, Kim's group[14] used a α, β- unsaturated carbonyl group containing chemodosimeter for the rapid detection of cyanide in analytical sample based on the fluorescence intensity. By combining indole and coumarin moieties Lee and Kim et al. able to develop new hybrid probe[15]

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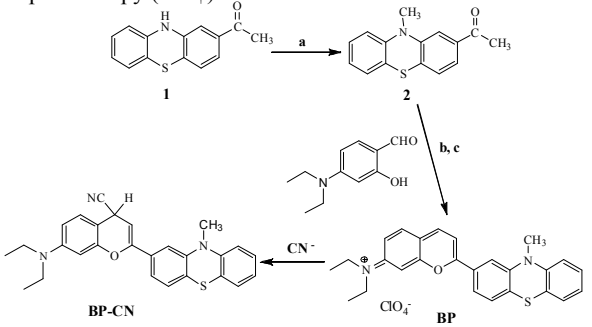
which selectively sense cyanide by not only chromogenic but also fluorogenic way. The response time was very short (<1 s) in acetonitrile–water (95:5) solvent medium. Chen et al. introduced dicyano-vinyl moiety,[16] which showed a large blue shift (96 nm) in the absorption spectra upon addition of cyanide to the probe. The resulted colour change could clearly be observe by the naked eye. They developed, the test strips based on this probe for practical and efficient detection of cyanide. As a part of our on-going research on ion sensing[17] and all these research studies[18] inspire us to develop a probe which could be used in reaction-based analytes sensing with a fast and sensitive fluorescence response.

Hence, reaction-based sensors displaying both colorimetric and fluorometric dual-channel responses to cyanide are able to offer quantitative information via their internal calibration of absorption and simultaneously to show the presence of cyanide via the altered color. With these considerations in mind, we designed and synthesized a novel reaction-based fluorometric and colorimetric sensor **BP** for cyanide anion based on a hybrid benzopyrylium-phenothizine-liked flavylium dye (Scheme 1). Such a molecular design makes probe **BP** possess the expanded π-conjugation as well as the strong ICT from the phenothizine to the diethylaminobenzopyrylium moiety, which will lead to the higher region absorption. Importantly, this type of compound can achieve high selectivity as well as naked-eye detection in a very short time because it possesses an electron-withdrawing benzopyrylium group that is highly reactive for cyanide. Therefore, the nucleophilic attack of cyanide toward the benzopyrylium group of probe **BP** will block the π -conjugation between phenothizine and diethylaminobenzopyrylium moiety, by which a hypsochromic shift of absorption and emission of phenothizine should be induced, resulting in the spectral changes as well as the change in color. These signal changes indicate that probe **BP** can serve as a selective chemosensor for cyanide anion.

## RESULTS AND DISCUSSION

Probe **BP** could be conveniently synthesized via the condensation of 4-(diethylamino)- salicylaldehyde with N-methyl-2-acetylthiophenazine in conc. $H_2SO_4$. Structural identification of the compound was confirmed by $^1$H NMR, $^{13}$C NMR, and ESI-MS spectroscopy (ESI†).




**Scheme 1** Synthesis of **BP**. Reagents and conditions: (a) MeI, $K_2CO_3$, KI, TBAB, Acetonitrile, (b) Conc. $H_2SO_4$, heat at 90°c , 6 h; (c) 70% $HClO_4$.

The sensitivity of probe **BP** toward different anions and their preferential selectivity toward $CN^-$ over the other anions has been studied by using UV-vis, fluorescence, and $^1$H NMR spectroscopic techniques.

**UV-vis and Fluorescence Spectral Behaviour of BP:** The sensitivity of probe **BP** toward different anions ($CN^-$, $AcO^-$, $F^-$, $Cl^-$, $Br^-$, $I^-$, $S^{2-}$, $H_2PO_4^-$, $SO_4^{2-}$, $NO_2^-$, $ClO_4^-$, $SCN^-$, $SO_3^{2-}$ and $CO_3^{2-}$) and biothios (Cys, Hcy and GSH) and their preferential selectivity toward $CN^-$ over the other ions has been studied by absorption and fluorescence titrations. The UV-vis spectrum of **BP** exhibited an absorption maximum at 552 nm with a small hump in the region at 450 nm in aqueous acetonitrile (3/7 v/v) HEPES buffer (pH 7.4). The band at 552 nm could be attributed to the intramolecular charge transfer transition from the donating phenothiazine ring to the diethylaminobenzopyrylium moiety which acts as an acceptor. Upon addition of increasing amounts of cyanide ($CN^-$) anion (as its n-$Bu_4N^+$ salt) (0–2 equiv.), the $\lambda_{max}$ of probe **BP** at 552 nm was decreased its intensity and another band at 450 nm was showed almost similar structural patterns. The same was recognized by the naked-eye in the form of color change from purple to light yellow (Fig. 1a, inset).

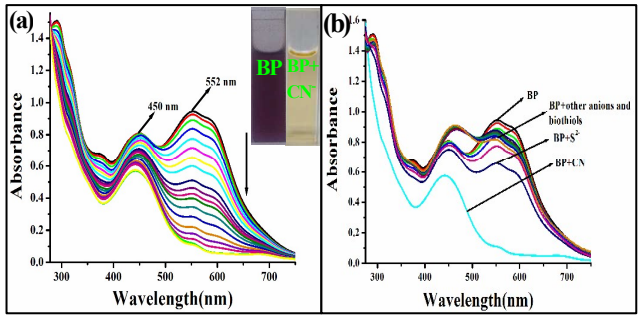



**Fig. 1** (a) Absorption titration curve of **BP** (c = $1\times10^{-5}$ M) in aq. $CH_3CN$ ($CH_3CN/H_2O$ = 7:3 v/v, 20 mM HEPES buffer, pH = 7.4) with $NBu_4CN$ (c = $1\times10^{-4}$ M). Inset shows naked eye color change before (purple) and after (light yellow) addition of $CN^-$ to **BP**. (b) Competitive absorption spectra of **BP** (c = $1\times10^{-5}$ M) in the presence of different anions (c = $1\times10^{-4}$ M) (n-$Bu_4N^+$ salts of $CN^-$, $AcO^-$, $F^-$, $Cl^-$, $Br^-$, $I^-$ and $Na^+$ salts of $S^{2-}$, $H_2PO_4^-$, $SO_4^{2-}$, $NO_2^-$, $ClO_4^-$, $SCN^-$, $SO_3^{2-}$ and $CO_3^{2-}$) and different biothios (c = $1\times10^{-4}$ M) (Cys, Hcy and GSH) in aq. $CH_3CN$ ($CH_3CN$:$H_2O$ = 7:3 v/v, 20 mM HEPES buffer, pH = 7.4).

Furthermore, the selectivity of **BP** for cyanide over other anions was examined. The results revealed that all potentially competitive anions exerted no or little influence (for $S^{2-}$) on the UV–vis detection of cyanide in aqueous acetonitrile (3/7 v/v) (Fig. 1b).

The most remarkable changes were seen in the fluorescence titration studies. The free probe **BP** exhibited weak emission at ~442 nm when excited at 350 nm in aqueous acetonitrile (3/7 v/v) mixture at pH 7.4 to have an effective 20 mM HEPES buffer solution. Titration of probe **BP** with $CN^-$ results in the enhancement of fluorescence intensity as a function of the added

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$CN^-$ concentration (Fig. 2a). With the addition of $CN^-$, the emission at 442 nm increased sharply, followed by the red shifted emission from 442 nm to 512 nm was observed (only N-methylphenothiazine moiety shows at 526 nm), indicating that the Michael addition reaction interrupted the π-conjugation and blocked the ICT process, after which the fluorescence of N-methylphenothiazine recovered. This is in good agreement with the aforementioned design concept. The fluorescence intensity increased linearly with added cyanide concentration (Fig. 2b). Addition of only 2 equiv of cyanide anion saturated the system, and no more change was seen with further addition of cyanide anion, suggesting 1:1 stoichiometric reaction of **BP** with cyanide anion; the corresponding Jobs plot provided additional support to the stoichiometric ratio (Fig. S6†). The fluorescence detection limit was 0.13 μM in HEPES solution, which was also much lower than that of the most reported fluorometric and colorimetric cyanide probes (Fig. S9†).[19]

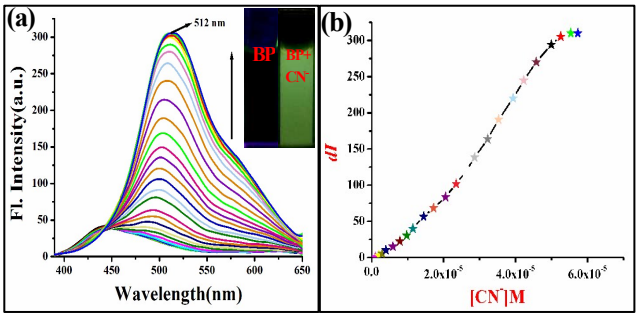


**Fig. 2** (a) Emission spectra of **BP** (c = $1\times10^{-5}$ M) ($\lambda_{ex}$ = 350 nm) in aq. $CH_3CN$ ($CH_3CN/H_2O$ = 7:3 v/v, 20 mM HEPES buffer, pH = 7.4) with $NBu_4CN$ (c = $1\times10^{-4}$ M). Inset shows fluorescent color change before (low intense blue) and after (bright green) addition of $CN^-$ to **BP**. (b) Change of emission intensity at 512 nm with incremental addition of $CN^-$ ions [$\lambda_{ext}$ = 350 nm].

The cyanide sensing process was also clearly seen not only by color change but also by bright green fluorescence under UV lamp. The color change from low intense blue to bright green fluorescence could be distinguished by the naked eyes as shown in Fig. 2a, inset.

**Interference of Other Anions Towards BP:** To evaluate the selectivity of **BP**, we measured the fluorescence intensity of **BP** in the presence of various anions ($CN^-$, $AcO^-$, $F^-$, $Cl^-$, $Br^-$, $I^-$, $S^{2-}$, $H_2PO_4^-$, $SO_4^{2-}$, $NO_2^-$, $ClO_4^-$, $SCN^-$, $SO_3^{2-}$ and $CO_3^{2-}$) and biothios (Cys, Hcy and GSH). The fluorescence maximum intensity of **BP** was dramatically increased almost 6 times after the addition of cyanide, whereas the fluorescence intensity changes were not observed by other anions but were just comparable to **BP** itself. Competitive assay also showed consistent evidence that **BP** only was transformed by cyanide anion. The fluorescence intensity of **BP** and other anions were restored as much as that of **BP** by addition of cyanide to the mixture of **BP** and other anions (Fig. 3b).

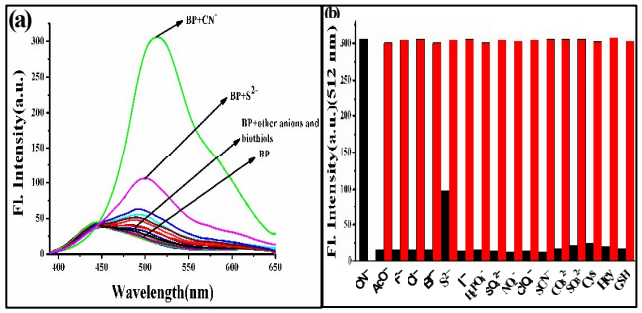


**Fig. 3** (a) Competitive fluorescence emission spectra of **BP** (c = $1\times10^{-5}$ M) in the presence of different anions (c = $1\times10^{-4}$ M) ($n$-$Bu_4N^+$ salts of $CN^-$, $AcO^-$, $F^-$, $Cl^-$, $Br^-$, $I^-$ and $Na^+$ salts of $S^{2-}$, $H_2PO_4^-$, $SO_4^{2-}$, $NO_2^-$, $ClO_4^-$, $SCN^-$, $SO_3^{2-}$ and $CO_3^{2-}$) and different biothios (c = $1\times10^{-4}$ M) (Cys, Hcy and GSH) in aq. $CH_3CN$ ($CH_3CN:H_2O$ = 7:3 v/v, 20 mM HEPES buffer, pH = 7.4). (b) Fluorescence response of **BP** (c = $1\times10^{-5}$ M) to addition of 2 equiv. $CN^-$ (c = $1\times 10^{-4}$ M) and 12 equiv. of other anions (c = $1\times10^{-4}$ M) [the black bar portion] and to the mixture of 12 equiv. of other anions (c = $1\times10^{-4}$ M) with addition of 2 equiv. $CN^-$ (c = $1\times 10^{-4}$ M) [the red bar portion].

These observations suggest that compound **BP** is highly selective toward $CN^-$ even in the presence of the complex mixture of anions.

**Time Response Experiments:** Time-dependent fluorescence spectra of **BP** were monitored in the presence of 2 equiv. cyanide (Fig. 4). The result revealed that the Michael type addition reaction can be finished within 30 s, indicating that the probe **BP** would provide a rapid detection of cyanide.

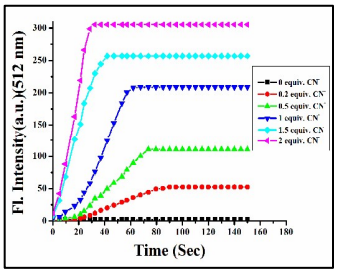


**Fig. 4** Variation of the fluorescence intensity at 512 nm *vs.* the reaction time of **BP** ($1\times10^{-5}$ M) in aq. $CH_3CN$ ($CH_3CN/H_2O$ = 7:3 v/v, 20 mM HEPES buffer, pH = 7.4) in the presence of different equiv. amount of $CN^-$ (c = $1\times 10^{-4}$ M).

**Fluorescence Response of BP at Various pH.** The titrations were carried out at different pH of the medium exhibited maximum fluorescence intensity in the pH ~ 7.4. However, at highly acidic and basic pH, the fluorescence of the **BP** during reaction with $CN^-$ shows a gradual decrease in intensity and the instability of the nucleophilic $CN^-$ under these conditions (Fig. S10†). This result indicates that the probe may be suitable for bio-applications at the physiological pH.

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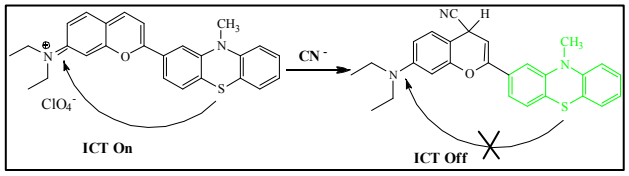
**Proposed Sensing Mechanism:** Such a molecular design makes probe **BP** possess the expanded π-conjugation as well as the strong ICT from the phenothizine to the diethylaminobenzopyrylium moiety, which will lead to the higher region absorption. Importantly, this type of compound can achieve high selectivity as well as naked-eye detection in a very short time because it possesses an electron-withdrawing benzopyrylium group that is highly reactive for cyanide. Therefore, the nucleophilic attack of cyanide toward the benzopyrylium group of probe **BP** will block the π-conjugation between phenothizine and diethylaminobenzopyrylium moiety, by which a decrease in intensity of absorption and emission of phenothizine should be induced, resulting in the spectral changes as well as the change in color (Scheme 2). These signal changes indicate that probe **BP** can serve as a selective chemosensor for cyanide anion.




**Scheme 2** Schematic presentation of nucleophilic attack of cyanide to **BP**.

**$^1$H-NMR Titration:** In addition, the binding pattern between probe **BP** and $CN^-$ was first examined by a $^1$H NMR titration experiment (Fig. 5). The $^1$H NMR titration studies showed that almost all aromatic proton signals shifted to up-field upon addition of $CN^-$ to the solution of **BP**; this is consistent with the proposed product in which the cationic diethylaminobenzopyrylium moiety is changed into a neutral one.

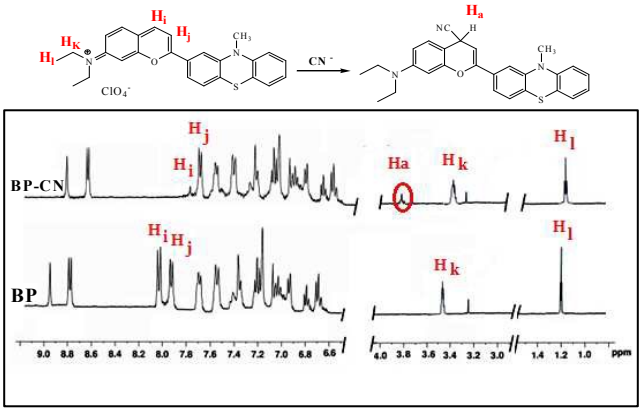



**Fig. 5** $^1$H NMR titration chart of **BP** (10.0 mg in 0.5 ml DMSO) without $CN^-$ and with addition of 2 equiv. of $CN^-$ in DMSO-d6 medium.

Especially, -$NCH_2$ ($H_K$) proton of diethylaminobenzopyrylium moiety shifted up-field from 3.46 ppm to 3.29 ppm, indicate the blocking of ICT process after the attack of $CN^-$ and hence produce the neutral molecule. In presence of $CN^-$, peak at 8.02 ppm (Hi) went nearly disappeared and a new peak was appeared at 3.78 ppm and peak at 7.92 ppm (Hj) of benzopyrylium moiety shifted up-field to 7.75 ppm. This result clearly indicate that C-4 carbon lost its unsaturation, supports the formation of **BP-CN** adduct.

**Theoretical Studies:** To investigate the electronic structure and the observed spectroscopic properties of the probe **BP** and its cyanide adduct **BP-CN**, DFT geometry optimizations followed by TD-DFT calculations using the Gaussian 09W[20] software package at the B3LYP/6-31G(*d,p*) + solv(PCM) level of theory were carried out. As well as to explain the highly reactive nature towards nucleophile of probe **BP**, the electrophilic Fukui function from density functional reactivity theory (DFRT)[21] was performed to estimate the electrophilicity of the reactive carbon centres such as C-2, C-4 and C-5 of chromene moiety in probe **BP** using the B3LYP exchange functional and the NBO charge. In general, electrophiles are molecules able to accept an amount of electron density along the reaction, nucleophiles are molecules able to donate an amount of electron density during the reaction.

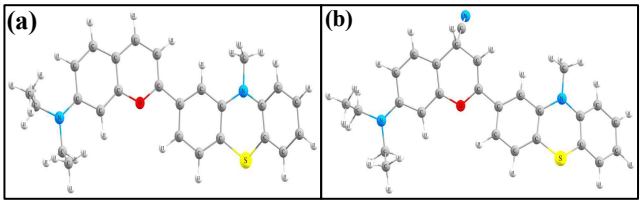



**Fig. 6** Calculated energy-minimized structure of (a) **BP** and (b) **BP-CN**.

From a theoretical point of view, the electrophilic and nucleophilic behaviours of organic molecules can be characterized by using the reactivity indices defined within the conceptual density functional theory (DFT) framework.[22] The calculations provide the electrophilicity of the carbon centers such as C-2, C-4 and C-5 as 3.0741, 4.4663 and 1.3922 au, respectively.

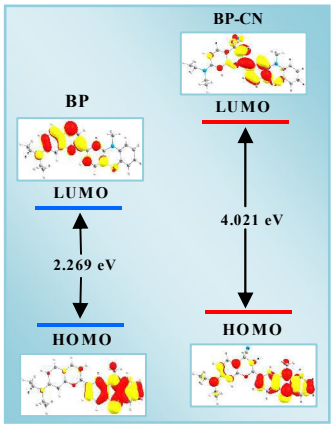



**Fig. 7** HOMO and LUMO distributions of **BP** and **BP-CN**, calculated at the B3LYP/6-31G(d) level.

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Thus, the C-4 carbon of probe **BP** is much more electrophilic than that of other centers C-2, and C-5, in good agreement with the observation that probe **BP** is much more reactive towards nucleophilic cyanide attack at C-4 centre instead of other centers. The optimized geometries of **BP** and **BP-CN** are shown in Fig. 6. The spatial distributions of frontier orbitals and orbital energies of HOMO and LUMO of **BP** and **BP-CN** were also determined (Fig. 7). The calculated HOMO-LUMO energy gaps of **BP** and **BP-CN** adduct is 2.269 and 4.021 eV respectively (Table S2†). There is a considerable difference in the energy minimisation structure in **BP** and **BP-CN** adduct, which can shed light on the change in spectral intensity in absorption spectra due to blockage of π-conjugation on $CN^-$ addition and the corresponding change in color. In **BP**, FMOs are mostly separated, HOMO is positioned along the electron-richer phenothiazine moiety, while the LUMO chiefly resides along the positive part of chromene moiety. This orbital distribution suggested that π-conjugation occurs at excited state and the cyanide-adduct of **BP** should impact HOMO and LUMO unevenly and thus constitute the basis of an optical response of **BP** to cyanide addition.

**Practical Application:** To investigate the practical application of probe **BP**, test strips were prepared by immersing filter papers into aqueous acetonitrile (3/7 v/v) mixture at pH 7.4 solutions of **BP** ($1.0\times 10^{-2}$ M) and then drying in air. The test strips containing **BP** were utilized to sense cyanide.

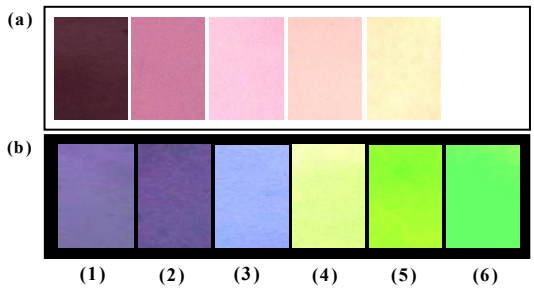


**Fig. 8** (a) Naked eye in visible light and (b) Fluorescence color changes visualized on filter paper strips of (1) **BP** ($c = 1.0\times 10^{-2}$ M) and during addition of $CN^-$ at (2) $1.0\times10^{-5}$ M; (3) $1.0\times10^{-4}$ M; (4) $1.0\times10^{-3}$ M; (5) $1.0\times10^{-2}$ M ; (6) $4.0\times10^{-2}$ M in aq. $CH_3CN$ ($CH_3CN/H_2O$ = 7:3 v/v).

As shown in Fig. 8, when cyanide was added on the test kits at various concentrations, the obvious color changes from burgundy to light yellow in visible light and from low intense blue to bright green under the 365 nm UV–lamp was observed. Therefore, the test strips coated with the probe **BP** solution would be convenient for detecting $CN^-$ according to guideline of WHO. These results showed that probe **BP** could have a practical application for detecting $CN^-$ in environmental samples.

**Live Cell Imaging:** To demonstrate the practical application of probe **BP** in biological sample, we performed a cellular imaging experiment of **BP** using A549 human cells under the confocal laser scanning microscope. As shown in Fig. 9, when A459 human cells were incubated with probe **BP** (2 μM) for 30 min at 37°C, a very weak fluorescence was observed. However, when A459 human cells were treated with probe **BP** followed by cyanide salts, we observed a bright green fluorescent from the intracellular area (in the green channel).

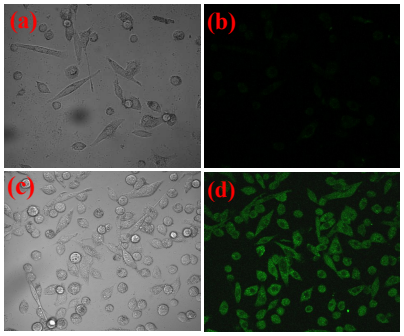


**Fig. 9** Confocal microscopic images of the probe **BP** in A549 human cells:(a) bright field image of the cells treated with probe **BP**, (b) confocal fluorescence image of probe **BP** at $2.0\times10^{-6}$ M concentration, (c) bright field image of the cells treated with probe **BP** and $NBu_4CN$ ($1.0\times10^{-5}$ M) (d) confocal fluorescence image of probe **BP** and $NBu_4CN$. all images were acquired with a 40× objective lens.

Appearance of such type of green fluorescence indicated the formation of **BP-CN** complex in the A549 human cells, which is also in good agreement with the fluorescence turn-on report of the probe in presence of cyanide ions in solution. Further, bright-field images of cells confirmed that the A549 human cells were viable throughout the imaging experiments. Thus, these results revealed that probe **BP** possesses good membrane permeability and is proficient for monitoring cyanide ions in living cells. In addition, we also studied MTT assay to know the cytotoxicity in A549 human cells and it is found that probe **BP** does not show noticeable cytotoxicity to A549 human cells (Fig. S11†).

**Conclusion:** In conclusion, we have synthesized and characterized a new colorimetric and fluorometric probe **BP** for $CN^-$ based on a hybrid benzopyrylium-phenothizine-liked flavylium moiety. Their selectivity and sensitivity were demonstrated on the basis of fluorescence, absorption, and $^1H$ NMR spectroscopy, and visual fluorescent color changes. The probe **BP** can detect $CN^-$ up to 0.13 μM, by switch-on fluorescence, suggesting its applicability to detect $CN^-$ ions in aqueous HEPES buffer medium. This work highlights a simple reaction mechanism between **BP** and cyanide anion. Furthermore, the test strips could conveniently and rapidly (within a short time of 30 s) detect cyanide in solutions. The probe **BP** was demonstrated as potential live-cell fluorescence imaging agents under microscopy using A549 human cells. Potential practical applications of the method are under intensive investigation in our laboratories.

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## Experimental Section

**General information and materials:** Unless stated otherwise, commercial grade chemicals were used for the synthesis of probes were purchased from Sigma Aldrich Chemical Co. (USA) and used without further purification. Salts of different anions viz. $CN^-$, $AcO^-$, $F^-$, $Cl^-$, $Br^-$ and $I^-$ as n-$Bu_4N^+$ salts and $S^{2-}$, $H_2PO_4^-$, $SO_4^{2-}$, $NO_2^-$, $ClO_4$ and $SCN^-$ as $Na^+$ salts were purchased from Spectrochem Pvt Ltd. (India), stored in a desiccators under vacuum containing self-indicating silica, and used without any further purification. All the solvents were of analytic grade. Solvents were dried according to standard procedures. Elix Millipore water was used throughout all experiments. All reactions were magnetically stirred and monitored by thin-layer chromatography (TLC) using Spectrochem GF254 silica gel coated plates. Column chromatography was performed with 60-120 silica gel. $^1H$ and $^{13}C$ NMR spectra were recorded on a Brucker 400 MHz, 300 MHz instrument. Mass spectra were carried out using a Waters QTOF Micro YA 263 mass spectrometer. For NMR spectra, DMSO-$d_6$ and $CDCl_3$ were used as solvent using TMS as an internal standard. Chemical shifts are expressed in δ ppm units and $^1H$–$^1H$ and $^1H$–C coupling constants in Hz. UV–visible and fluorescence spectra measurements were performed on a SHIMADZU UV-1800 and a PerkinElmer LS-55 spectrofluorimeter respectively. The following abbreviations are used to describe spin multiplicities in $^1H$ NMR spectra: s = singlet; d = doublet; t = triplet; m = multiplet.

**Preparation of test solution for UV−vis and fluorescence spectral studies:** A stock solution of the probes **BP** ($1.0\times10^{-5}$ M) were prepared in $CH_3CN/H_2O$ (7:3 v/ v). Solutions of $1.0\times10^{-4}$ M salts of the respective anions were prepared in Millipore water. All experiments were carried out in $CH_3CN/H_2O$ solution ($CH_3CN/H_2O$ = 7:3 v/v, 20 mM HEPES buffer, pH = 7.4). In titration experiments, each time a $1\times10^{-5}$ M solution of **BP** were filled in a quartz optical cell of 1 cm optical path length, and the ion stock solutions were added into the quartz optical cell gradually by using a micropipet. Spectral data were recorded at 1 min after the addition of the ions. In selectivity experiments, the test samples were prepared by placing appropriate amounts of the anions ($1.0\times10^{-4}$ M) stock into 2 mL of solution of **BP** ($1\times10^{-5}$ M).

**Detection limit studies :** The detection limit was calculated on the basis of the fluorescence titration. The fluorescence emission spectrum of **BP** was measured 10 times, and the standard deviation of blank measurement was achieved. To gain the slope, the ratio of the fluorescence intensity at 512 nm was plotted as a concentration of $CN^-$. So the detection limit was calculated with the following equation.

$$\text{Detection limit} = 3Sb1/S \qquad (1)$$

where Sb1 is the standard deviation of blank measurement and S is the slope of the calibration curve.

**Computational studies:** All geometries for **BP** and **BP-CN** were optimized by density functional theory (DFT) calculations as well as time-dependent density function theory (TDDFT) calculations were performed with Gaussian09 program package, with the aid of the GaussView visualization program.

**Cell incubation studies:** A549 human cell (ATCC: CCL-185) lines were prepared from continuous culture in Dulbecco's modified Eagle's medium (DMEM, Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum (Invitrogen), penicillin (100 μg/mL), and streptomycin (100 μg/mL). The A549 human cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in DMEM containing 10% (v/v) fetal bovine serum and antibiotics in a $CO_2$ incubator. Cells were initially propagated in 75 $cm^2$ polystyrene, filter-capped tissue culture flask in an atmosphere of 5% $CO_2$ and 95% air at 37°C in $CO_2$ incubator. When the cells reached the logarithmic phase, the cell density was adjusted to $1.0\times10^5$ per/well in culture media. The cells were then used to inoculate in a glass bottom dish, with 1.0 mL ($1.0\times10^4$ cells) of cell suspension in each dish. After cell adhesion, culture medium was removed. The cell layer was rinsed twice with phosphate buffered saline (PBS), and then treated according to the experimental need.

**Cellular imaging methodology:** For confocal imaging studies A549 human cells, $1\times10^4$ cells in 1000 μL of medium, were seeded on sterile 35 mm covered Petridis, glass bottom culture dish (ibidi GmbH, Germany), and incubated at 37°C in a $CO_2$ incubator for 10 hours. Then cells were washed with 500 μL DMEM followed by incubation with 1.0 x $10^{-5}$ M $NBu_4CN$ dissolved in 500 μL DMEM at 37°C for 1 h in a $CO_2$ incubator and observed under an Olympus IX81 microscope equipped with a FV1000 confocal system using 1003 oil immersion Plan Apo (N.A. 1.45) objectives. Images obtained through section scanning were analyzed by Olympus Fluoview (version 3.1a; Tokyo, Japan) with excitation at 395 nm monochromatic laser beam, and emission spectra were integrated over the range 350-650 nm (single channel). The cells were again washed thrice with phosphate buffered saline PBS (pH 7.4) to remove any free $NBu_4CN$ and incubated in PBS containing probe **BP** to a final concentrations of $2.0\times10^{-6}$ M, incubated for 10 min followed by washing with PBS three times to remove excess probe outside the cells and images were captured. In separate culture dish the cells were similarly treated with $2.0\times10^{-6}$ M probe **BP** incubated for 10 min, washed thrice with PBS and the image was captured to get any possible background fluorescence.

**Preparation of compound 2:** 2-acetylthiophenazine (300 mg, 1.24 mmol) was dissolved in dry $CH_3CN$. $K_2CO_3$ (257 mg, 1.86 mmol), a pinch of KI and catalytic amount TBAB were added to the solution. Drop wise methyl iodide (265 mg, 1.86 mmol) was added into it and the reaction mixture was stirred at 82 °C for overnight. After reflux, the solvent was evaporated in vacuum. Afterwards, the mixture was extracted with dichloromethane. The extractions were combined, washed with distilled water, saturated brine, and then dried over anhydrous sodium sulfate. After

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evaporation of solvent, the crude compound was purified over silica gel column and desired compound was eluted by PE: EA (10:1 v/v) to obtain 210 mg pure product as yellow liquid. Yield: 66%. Mp above 250°C. $^1$H-NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.35 (d, 1H, J=7.8 Hz), 7.11 (s, 1H), 6.96 (q, 3H), 6.81 (1H, t, J=7.16 Hz), 6.53 (d, 1H, J=7.64 Hz), 5.95 (s, 1H), 3.29 (s, 3H), 2.51 (s, 3H). MS (ESI-MS): (m/z, %): 256.40 [M+H]+; Calculated for $C_{14}H_{11}NSO$: 255.25.

**Preparation of BP**: 4-diethylamino-salicylaldehyde (0.151 g, 0.784 mmol) and compound 2 (0.2 g, 0.784 mmol) were dissolved in conc. $H_2SO_4$ (7 ml). The reaction mixture was stirred at 80-90$^o$C temperature for 6 h. After the completion of the reaction (monitored by TLC), the reaction mixture was poured into ice-water, and then 70 % perchloric acid (2 ml) were added; then deep purple solid precipiteted was filtered through suction, washed several times with milipore water and dried in air. The residue was purified *via* chromatography with silica gel ($CH_3Cl$ : $CH_3OH$ = 95.5 : 0.5) and solid purple product was obtained (0.342 g, 93 % yield); M.P. > 250°C. $^1$H NMR (DMSO- $d_6$, 400 MHz) δ(ppm): 8.9475 (s, 1H), 8.7801 (d, 1H, J= 8.0 Hz), 8.02985 (d, 1H, J= 9.48 Hz), 7.92595 (d, 1H, J= 7.8 Hz), 7.6764 (t, 1H, J= 7.32 Hz), 7.5443 (d, 1H, J= 9.6), 7.3555 (d, 1H, J= 8.72 Hz), 7.1625 (s, 1H), 7.033 (q, 1H), 6.94 (d, 1H, J= 7.32 Hz), 6.79 (t, 1H, J= 7.28 Hz), 3.467325 (q, 4H), 3.2588 (s, 3H), 1.193 (t, 6H, J= 8.8 Hz),. $^{13}$C NMR (DMSO-$d_6$, 75 MHz) δ(ppm): 159.79, 142.39, 142.07, 141.72, 141.14, 138.64, 131.64, 129.07, 128.27, 127.47, 124.54, 123.16, 122.39, 122.08, 115.78, 114.93, 114.52, 113.30, 110.14, 104.74, 97.71, 44.42, 42.49, 12.93. TOF MS ES+, m/z = 414.4 $[M+H]^+$; calculated for $C_{26}H_{25}N_2SO$ = 413.168.

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**Notes and references**

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# Table of Contents

## Benzopyrylium-phenothiazine-conjugate of flavylium derivative as fluorescent chemosensor for cyanide in aqueous media and its bioimaging

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