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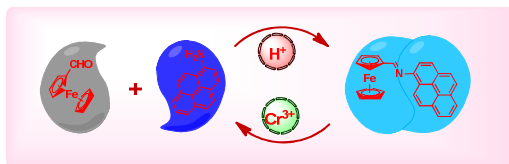


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Table of Contents entry**A ferrocene-pyrene based ‘turn-on’ chemodosimeter for Cr³⁺ - Application in Bioimaging****Mandeep Kaur,^a Paramjit Kaur,^{*a} Vikram Dhuna,^b Sukhdev Singh,^b Kamaljit Singh^{*a}**

Structurally simple, ferrocene-pyrene imine dyad **1** detects Cr³⁺ *via* the metal ion triggered hydrolysis of imine functionality with subsequent ‘turn-on’ fluorescence behaviour. Further, the non-cytotoxic dyad detects Cr³⁺ in the human breast cancer (MCF-7) cells.

ARTICLE

A ferrocene-pyrene based 'turn-on' chemodosimeter for Cr³⁺ - Application in Bioimaging

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Structurally simple, ferrocene-pyrene imine dyad **1** has been developed as a 'turn-on' chemodosimeter for Cr³⁺. The sensing event is based upon the hydrolysis of the imine functionality. Further, **1** which is also non-cytotoxic (100% cell viability) detects intracellular Cr³⁺ in the human breast cancer (MCF-7) cells.

Introduction

Chromium, one of the most common elements in the earth's crust and seawater, exists principally as metallic (Cr⁰), trivalent (+3), and hexavalent (+6) forms. While the more common trivalent chromium is essential and found in most food and nutrient supplements, the hexavalent chromium is highly toxic. The trivalent chromium has profound impact on glucose metabolism and is implemented to counter diabetes, cardiovascular and nervous system disorders etc.¹ Exposure to high levels of Cr³⁺ can also have adverse effect on the normal enzymatic activities and cellular structures.² Further, the release of excess of Cr³⁺ poses serious threat to the environment.³ In the absence of simple methods, development of chemosensors for Cr³⁺, relying specifically on cost-effectiveness, efficiency and biocompatibility could provide a viable alternative to the often intricate and expensive methods based on inductively coupled plasma mass spectrometry, atomic absorption etc.⁴ Owing to the tendency of the paramagnetic Cr³⁺ to quench fluorescence emission,⁵ generally a "turn-off" response is observed in sensing events, there are very limited reports⁶

wherein a 'turn-on' response has been reported during sensing of Cr³⁺. We have in the past reported⁷ donor-acceptor dyads for metal ion sensing which showed variable optical responses upon detecting different analytes. Usually, a donor (D) of ferrocene type turns the fluorescence of pyrene 'off' when used as an acceptor (A) in the D-A dyads through PET or related photo-physical processes as discussed later in this paper. Upon interrupting the quenching process, the fluorescence, if 'turn-on', furnishes efficient fluorescent probe for different analytes. We previously reported a dyad of ferrocene and 2-(3,5,5-trimethylcyclohex-2-enylidene)malononitrile for the efficient detection of Cu²⁺ which acted through complexation of Cu²⁺ with ferrocene as well as the alkene bridge.^{7c} The linker alkene provided an additional coordination site to the metal ion which as a consequence caused electronic perturbation in the D-A receptor leading to the sensing process. However, in the present case, as we describe below in case of a new D-imine-A dyad, the imine functionality breaks in a metal triggered hydrolysis reaction in the presence of Cr³⁺ and leads to the application of this dyad as efficient chemodosimeter. Similar chemodosimeters proceeding through hydrolysis reaction have received some attention in the past.⁸ However, in such reports, either the detection limit and/or the time required for the completion of the process has not been mentioned, which constitute important parameters of a detection process.^{8a,c}

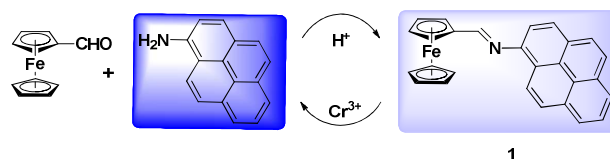
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†Electronic Supplementary Information (ESI) available: [Supporting Information (see footnote on the first page of this article): Spectral data of **1**, contour surfaces, TD-DFT data, cartesian co-ordinates and complete reference no. 12] See DOI: 10.1039/b000000x/



Scheme 1 Synthesis and Cr³⁺ induced hydrolysis of chemodosimeter **1**.

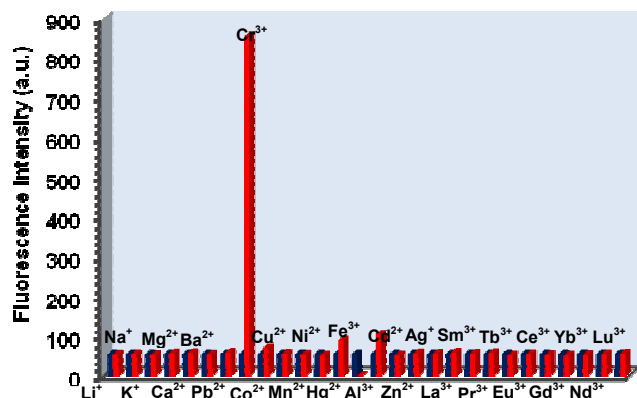


Fig. 1 Changes in the emission properties of **1** (5.0×10^{-6} M, in THF) (blue) at 442 nm, upon addition of various metal ions (5.0×10^{-4} M, in water) (red). (The v/v ratio of THF and H₂O in the mixture was 1:99).

In continuation of our research interest, we report the synthesis and chemodosimeter behaviour of a structurally simple and new dyad (*1E*)-*N*-(ferrocenylmethylene)pyren-1-amine **1** (Scheme 1), which unequivocally depicted efficient fluorescence 'turn-on' chemodosimetric response in the presence of Cr³⁺ in comparison to competitive metal ions (Fig. 1). To the best of our knowledge, **1** constitutes an exclusive example of a simple, ferrocene-imine-pyrene chemodosimeter for the detection of Cr³⁺. We also furnish robust evidences that the sensing event operates *via* a metal ion promoted hydrolysis of imine functionality of **1**, as the by-products: 1-aminopyrene and 1-ferrocenecarboxaldehyde have been isolated from the sensing experiment as depicted in Scheme 1. The most attractive feature of **1** is the fact that it exhibits rarely observed 'turn-on' behaviour in the presence of paramagnetic Cr³⁺.

Results and discussion

1. Synthesis and characterization

Chemodosimeter **1** was synthesized in one step in 74% yield *via* the simple acid catalysed Schiff-base condensation reaction of 1-ferrocenecarboxaldehyde and 1-aminopyrene (Scheme 1). **1** depicted satisfactory spectroscopic [¹H and ¹³C NMR, ESI-MS, FT-IR (Fig. S1-S4, see †ESI)] and microanalytical data.

2. Behaviour of **1** towards cations

The fluorescence spectrum of **1** (5×10^{-6} M in THF, $\lambda_{\text{exc.}} = 350$ nm) is characterized by a weak emission band peaking at 445 nm attributed to the monomer emission⁹ of **1** albeit with low quantum yield ($\Phi = 0.016$) which is characteristic¹⁰ of the quenched emission of pyrene *via* either photoinduced electron transfer (PET) or energy transfer from the ferrocenyl-imine unit acting as electron donor (steps i-iii, Fig. 2) to the excited pyrenyl subunit acting as electron acceptor.¹¹ It was found that among a number of metal ions tested (Fig. 1), Cr³⁺ enhanced both, the intensity as well as quantum yield ($\Phi = 0.528$) (33 fold) of the emission band owing to the formation of 1-aminopyrene (step iv, Fig. 2), compared to the free dyad **1**. As 1-ferrocenecarboxaldehyde and 1-aminopyrene have indeed been isolated (see †ESI) experimentally, it is proposed that the

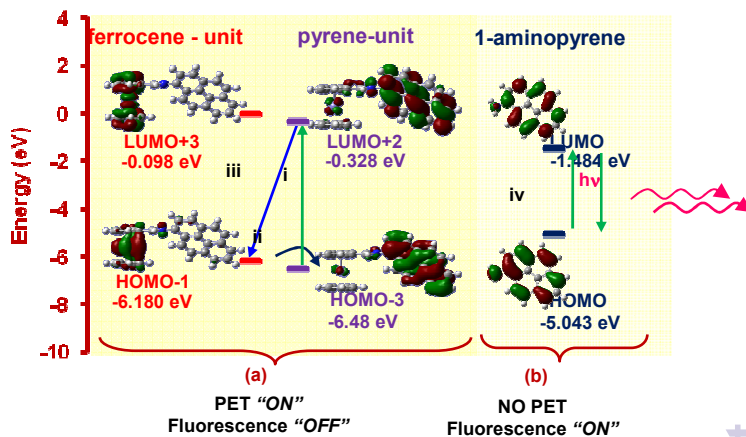


Fig. 2 HOMO-LUMO energy levels of (a) **1** and (b) free 1-aminopyrene.

Cr³⁺ mediated hydrolysis of **1** leads to the formation of 1-aminopyrene due to which the fluorescence is turned 'on'. It is reasonable to assume that Cr³⁺ species which otherwise quench fluorescence would also be present in the solution, we found that compared to the higher fluorescence intensity and the quantum yield ($\Phi = 0.580$) of the free 1-aminopyrene, the quantum yield ($\Phi = 0.526$) decreases and matches with the value obtained in the present process.

The fluorometric titrations were conducted by adding the increased concentrations of Cr³⁺ ions (2.85×10^{-6} M to 5.0×10^{-4} M, in H₂O) to a solution of **1** (5.0×10^{-6} M, in THF). A gradual increase in the intensity of the emission band centred at 442 nm was observed (Fig. 3). It is pertinent to mention that the increase in fluorescence continued until the addition of Cr³⁺ ions (5.0×10^{-5} M, in H₂O) with the detection limit of 1 μ M (Fig. S5, see †ESI), but the process completed only after 30 min. of equilibration. However, when a higher concentration of Cr³⁺ (5.0×10^{-4} M, in H₂O) was employed, a similar increase in the fluorescence intensity could be achieved much faster (<10 min., Fig. 3 inset). Literature records detection of metal ions, where the detection times ranging from 5 to 60 min. have been reported upon addition of 10-50 equiv. of the metal ions.^{8b,c}

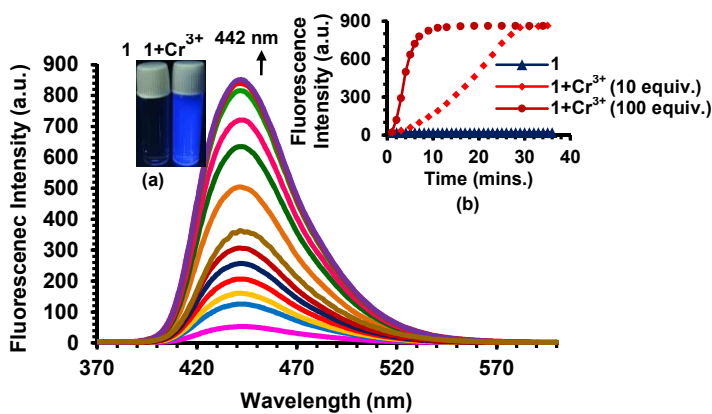


Fig. 3 Changes in the emission spectra of **1** (5.0×10^{-6} M, in THF) recorded after five min. of addition of solution of Cr³⁺ (2.85×10^{-6} M to 5.0×10^{-4} M, in H₂O) (overall THF:H₂O ratio, 1:99 v/v). Inset: (a) Visual change in fluorescence of **1** upon addition of 5.0×10^{-4} M Cr³⁺, (b) Revival of fluorescence of **1** in the presence of 10 and 100 equiv. of Cr³⁺.

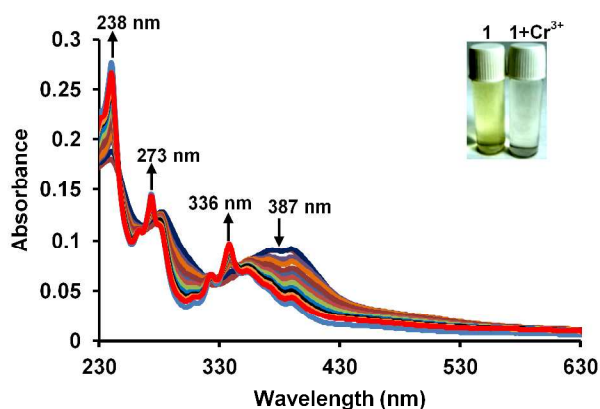


Fig. 4 Changes in the absorption spectra of **1** (5.0×10^{-6} M, in THF) upon addition of solution of Cr^{3+} (2.8×10^{-5} M to 5.0×10^{-4} M, in H_2O) (red). (The v/v ratio of THF and H_2O in the mixture was 1:99). Inset: (a) Visual change in color of **1** upon addition of 5.0×10^{-4} M Cr^{3+} .

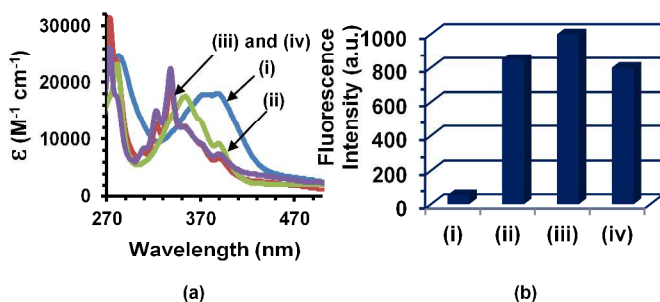


Fig. 5 (a) Absorption spectra and (b) emission profile (at 442 nm) of (i) **1**, (ii) 1-aminopyrene, (iii) **1** in the presence of 100 equiv. of Cr^{3+} , and (iv) 1-aminopyrene in the presence of Cr^{3+} , superimposed by iii in (a).

The UV-vis. absorption spectrum of **1** (5.0×10^{-6} M, in THF) is characterized by a broad split band centred at 387 ($17800 \text{ M}^{-1} \text{ cm}^{-1}$) and two high energy bands at 283 and 238 nm (14800 and $22600 \text{ M}^{-1} \text{ cm}^{-1}$ respectively) (Fig. 4). A low intensity broad band at 474 nm ($4000 \text{ M}^{-1} \text{ cm}^{-1}$) was also observed which has major contribution of the ferrocene based H-2→L+3 transition as deduced from Gaussian 09 suite of programmes.¹²

As the participating orbitals are located on the Fe atom and the cyclopentadienyl (Cp) moieties of ferrocene, it is assigned as a $\text{Fe}^{2+} \rightarrow \text{Cp}$ transition. Further, the split band at 387 nm has main contribution of HOMO (H)→LUMO (L) transition, with relatively little contribution from the H→L+1 transition. The HOMO is mainly located on the pyrene, while the LUMO and the associated L+1 are also mainly located on pyrene as well as the imine π -bridge, the transition (H→L+1) corresponds mainly to the pyrene chromophore. The high energy transitions (273 and 238 nm) were assigned as intra-ligand transitions of **1**, based on the contributing orbitals (Fig. S6 and Table S1-S2, see †ESI).

Upon addition of aqueous solution (5.0×10^{-4} M, in H_2O) of Cr^{3+} ion to a solution of **1** (5.0×10^{-6} M, in THF), while the band at 474 nm observed insignificant changes (Fig. 4) in intensity, the split bands centred at 387 nm observed both, a

decrease in intensity as blue shift. All these absorption changes were accompanied by appearance of new band at 336 nm, the position as well as shape of which matched with the absorption spectrum of 1-aminopyrene in the presence of Cr^{3+} (Fig. 5a), recorded independently. A similar correlation could be observed in the emission profile also (Fig. 5b). Thus, the observed absorption and emission changes established the formation of 1-aminopyrene, giving support to the proposed hydrolysis of **1** (Scheme 1) in the sensing event.

The hydrolytic cleavage of **1** into its constituting precursors was further confirmed by recording ^1H NMR spectrum of **1** in the presence of Cr^{3+} (1.0 equiv.) after equilibration, whereupon the signals corresponding to the hydrolytic products were clearly visualized (Fig. S7, see †ESI).

Based on the literature precedence,^{8b} it is reasonable to assume that binding of metal ion to the imine functionality may trigger its hydrolysis, to release 1-aminopyrene and 1-ferrocene carboxaldehyde in solution, leading to chemodosimeter action of **1**. Further, the spectroscopic changes (fluorescence as well as absorbance) were not reversed upon addition of EDTA solution (Fig. 6b), which is also expected for chemodosimeters.

To rule out the possibility of alternate mode of sensing through the formation of 1:Cr^{3+} complex, when an independent reaction of **1** with $\text{Cr}(\text{ClO}_4)_3 \cdot 6\text{H}_2\text{O}$ was performed, we could only observe the formation of 1-ferrocenecarboxaldehyde and 1-aminopyrene (TLC, ^1H NMR, (Fig. S8) mass spectra (Fig. S9), see †ESI) and no Cr^{3+} complex could be isolated.

To check any interference by competitive metal ions, when the titration of Cr^{3+} was repeated in the presence of a number of metal ions (*vide experimental*), no interference was observed

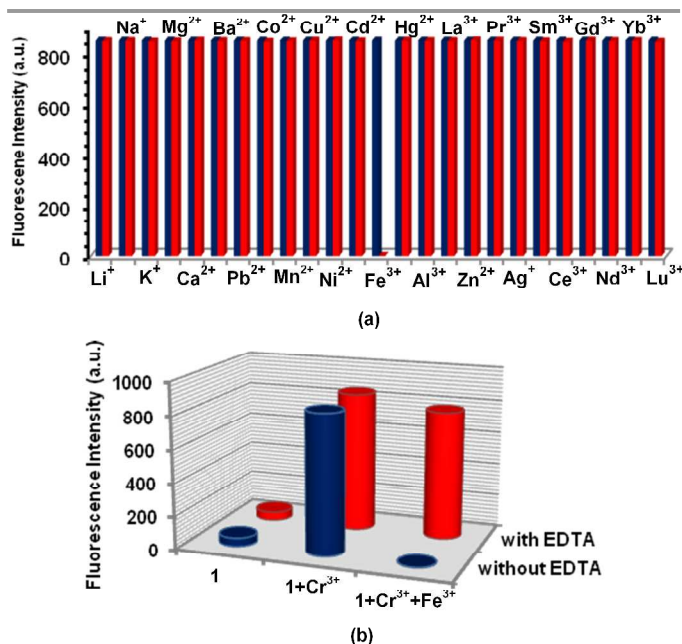


Fig. 6 (a) Emission of **1** (5.0×10^{-6} M, blue) in the presence of Cr^{3+} (5.0×10^{-4} M) and emission of **1** upon addition of Cr^{3+} (5.0×10^{-4} M, red) in the presence of other cations (5.0×10^{-4} M) in THF: H_2O (1:99/v/v); (b) Emission properties of **1**, 1+Cr^{3+} and $\text{1+Cr}^{3+}+\text{Fe}^{3+}$ in the presence (red) and in the absence (blue) of 2.5 equiv. of EDTA, in THF: H_2O (1:99/v/v) (at 442 nm).

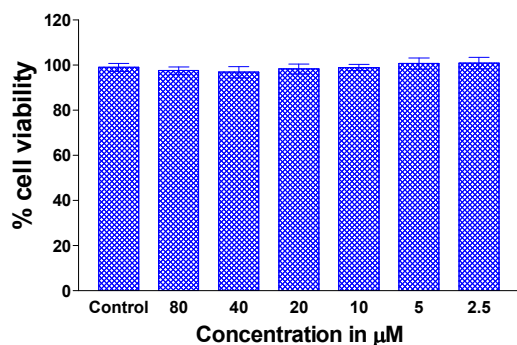


Fig. 7 MTT assay of breast cancer MCF7 cells cultured for 24 hours in media containing various concentrations of **1**.

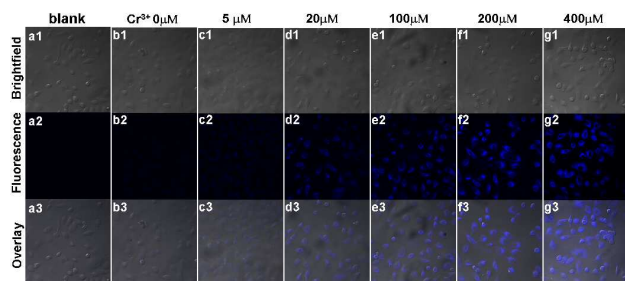


Fig. 8 Confocal images of MCF-7 cells, supplemented with varying concentration of $\text{Cr}(\text{ClO}_4)_3$ and **1** (5.0×10^{-6} M) (λ_{ex} 405 nm). a1-a3 were reference cells without **1**.

(Fig. 6a), except Fe^{3+} which quenched the emission intensity like many transition metals.¹³ This might in fact limit the applicability of **1** to sense Cr^{3+} in those situations where Fe^{3+} is a contaminant and need to be removed through precipitation (Fig. 6b) or other methods.¹⁴

Further, the effect of pH variation on **1** was also noted by recording the fluorescence spectra (Fig. S10, see †ESI). Only minor change was observed in the emission band of **1** in the pH range of 5-12 suggesting that **1** could be used in physiological environment where the pH is >5 without resorting to the buffered media. However, an increase in the emission intensity with decreasing pH may also be attributed to the hydrolysis of the imine functionality as the Schiff-bases are known to undergo acid promoted hydrolysis.¹⁵

3. Application in cell imaging

In line with the reports,¹⁶ relating the role of heavy metal ions (including Cr^{3+}) to cell malignancy in breast cancer cells MCF-7, we employed **1** as an imaging agent for the detection of Cr^{3+} in MCF-7 cells. Interestingly, no cytotoxic effect was revealed even at higher dose (80 μM) used in MTT assay¹⁷ (Fig. 7), indicating that **1** could be employed as a highly viable chemosensor in biomedical research aimed at exploring the bioactivity of Cr^{3+} in biological systems.

Fig. 8 shows the confocal microscope images of MCF-7 cells treated with different concentrations of Cr^{3+} . The fluorescence was particularly visible in the perinuclear region of the cells as suggested by the overlay (Fig. 8a3-8g3) of

fluorescence and bright field images indicating the subcellular distribution and excellent membrane permeability of **1**.

Conclusions

In conclusion, we have disclosed the 'turn-on' fluorescent-based selectivity of a new pyrene-imine-ferrocene based dyad **1** for Cr^{3+} . The dyad **1** constitutes a simple and inexpensive chemodosimeter which demonstrates a highly viable and useful application for the detection of Cr^{3+} in solution as well as in biological samples.

Experimental

1. Materials and general methods

Metal salts used in the spectrophotometric studies were of analytical grade and bought from Sigma-Aldrich. The solvents used were of analytical grade and purchased from Thomas Baker. Tetrahydrofuran (THF) was dried over sodium and benzophenone as an indicator. UV-vis. and fluorescence studies were performed in dry THF and double distilled water. Biological cell imaging was done in 0.1 M PBS solution (pH = 7.2). Stock solutions (0.1 M) of perchlorate salts of Li^+ , Na^+ , Mg^{2+} , Ca^{2+} , Pb^{2+} , Ba^{2+} , Mn^{2+} , Co^{2+} , Ni^{2+} , Cu^{2+} , Hg^{2+} , Cr^{3+} , Fe^{3+} and nitrate salts of K^+ , Ag^+ , Zn^{2+} , Cd^{2+} , Al^{3+} , La^{3+} , Ce^{3+} , Pr^{3+} , Sm^{3+} , Gd^{3+} , Yb^{3+} , Nd^{3+} , Eu^{3+} , Tb^{3+} , Lu^{3+} ions were prepared in doubly distilled water. Stock solutions of dyad **1** (1×10^{-3} M) were prepared in THF. Test solutions (5.0×10^{-6} M) were prepared by taking 17.5 μM of the stock solution of dyad **1** and diluting the solution to 3.5 mL with water, and adding an appropriate aliquot of each metal stock (17.5 μM of 0.1 M stock solution to prepare 5.0×10^{-4} M).

IR spectra were recorded on a Perkin Elmer Spectrum Two-IR Fourier-Transform spectrophotometer in the range 400-4000 cm^{-1} using KBr as medium. ^1H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectra were recorded in CDCl_3 on a JEOL-FT NMR-AL spectrophotometer. Tetramethylsilane (SiMe_4) served as the internal standards and CDCl_3 (7.26 ppm for ^1H and 77.0 ppm for ^{13}C) was used as solvent. Data are reported as follows: chemical shift in ppm (δ), multiplicity (s = singlet, m = multiplet), integration and interpretation. Mass spectra were recorded on a Bruker LC-MS microTOF II spectrometer. The purity of the products was checked by elemental analysis performed on a Thermoelectron FLASH EA1112 CHNS analyzer and were within $\pm 0.4\%$ of the theoretical values. Melting points were determined in open capillaries and are uncorrected. The fluorescence spectra were recorded on Perkin Elmer LS 55 spectrofluorimeter with excitation slit width as 15.0 and emission slit width as 2.5. UV-vis. spectra were recorded on a SHIMADZU 1601 PC spectrophotometer, with a quartz cuvette (path length, 1 cm) and studies were performed in AR grade THF and double distilled water. The cell holder of the spectrophotometer was maintained at 25°C for consistency in the recordings. The biological cell imaging was carried out on the NIKON A1R confocal microscope.

2. Synthesis of (1E)-N-(ferrocenylmethylene)pyren-1-amine (1)

To a solution of 1-pyreneamine (217 mg, 1.0 mmol) in ethanol (15 mL) added 2 drops of formic acid. To this added a solution of 1-ferrocenecarboxaldehyde¹⁸ (214 mg, 1.0 mmol) in ethanol (15 mL) under nitrogen atmosphere. Stir the reaction mixture on room temperature for one hour. Filter the resulting solid and recrystallize in Ethanol: Chloroform (3:1) mixture to afford **1** as a dark red solid (384 mg, 74%); mp: >200 °C. IR (KBr): $\nu_{\max}/\text{cm}^{-1}$ 3040 (aromatic C-H), 1615 (pyrene C=C), 1590 (C=N), 1104 (Cp). ¹H NMR (300 MHz, CDCl₃, 25 °C): δ 4.34 (s, 5H, CpH), 4.58 (s, 2H, CpH), 4.99 (s, 2H, CpH), 7.64 (s, 1H, N=CH), 7.96-8.17 (m, 7H, Aromatic CH), 8.57 (s, 2H, Aromatic CH) ppm. ¹³C NMR (75 MHz, CDCl₃, 25 °C): 69.212, 69.393, 71.478, 80.865, 122.823, 122.955, 123.227, 124.208, 124.694, 124.909, 125.576, 126.054, 126.236, 126.895, 127.365, 127.909, 131.568, 131.873, 134.576, 162.309 ppm. MS (EI): m/z 414.09 (M⁺+1). Anal. Calcd. (%) for C₂₇H₁₉NFe (413.09): C, 78.45; H, 4.60; N, 3.39. Found: C, 78.32, H, 4.51, N, 3.33. (Fig. S1-S4, see †ESI).

3. Quantum yield calculations

The fluorescence quantum yields were determined by using optically matching solution of 9,10-diphenylanthracene as standard having quantum yield of 0.86 in cyclohexane¹⁹ using the equation:

$$\Phi_x = \Phi_{st} \times (F_x \cdot A_{st} \times \eta_x^2) / (F_{st} \cdot A_x \times \eta_{st}^2)$$

Where Φ_x and Φ_{st} are the quantum yields of the test sample and the standard sample respectively, A_x and A_{st} are the absorbance of the test sample and the standard sample respectively, F_x and F_{st} are areas of emission bands for test sample and reference sample, η_x and η_{st} are the refractive indices of test sample and standard sample solutions in their respective pure solvents.

4. Computational Methods

All theoretical calculations were carried out by using the Gaussian 09 suite of programs. The molecular geometries of the chromophores were optimized at the Density Functional Theory (DFT) calculations employing the hybrid B3LYP²⁰ functional. 6-31G* basis set was used for C and H, aug-cc-pVTZ for donor N atom and LANL2DZ with effective core potential for the metal atom Fe. The frequency calculations were also performed using the same method and basis sets. The frequency analysis indicates that the optimized geometry is true energy minima because no imaginary frequency is found. Energy values and properties of the systems were computed considering solvent (water) effects by using the Cossi and Barone's CPCM (conductor-like polarizable continuum model) modification²¹ of the Tomasi's PCM formalism.²² The first 30 excited states were calculated by using Time-Dependent Density Functional Theory (TD-DFT calculations). The molecular orbital contours were plotted using Gauss view 5.0.9.

5. Cytotoxicity assay

To check that the cytotoxic effect of **1**, MCF-7 cells were seeded at 3×10^3 cells/well in 100 μ l DMEM containing 10% FBS in 96-well tissue culture plate and incubated for 48 h at 37°C (till 50% confluence), 5% CO₂ in air and 90% relative humidity in CO₂ incubator. After incubation, 100 μ l of **1** solution (80, 40, 20, 10 and 5 μ M), prepared in DMEM, was added to cells and the cultures were incubated for 24 hours. Four hours before the termination of experiment, the medium was discarded and 100 μ l DMEM containing MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (5 mg/ml) was added to the cells and incubated in CO₂ incubator at 37°C in dark for 4 hours. After incubation, the purple colored formazan produced in the cells appeared as dark crystals in the bottom of the wells. The culture medium was aspirated from each well carefully to prevent disruption of the cell monolayer. At last, 100 μ l of DMSO was added in all wells and mixed thoroughly to dissolve the formazan crystals, producing a purple solution. The absorbance of the 96 wells plate was taken at 570 nm with Labsystems Multiskan EX ELISA reader against a reagent blank. The cytotoxic effect of each treatment was expressed as percentage of cell viability relative to the untreated control cells.

6. Live cell imaging studies

Human breast adenocarcinoma MCF-7 cell line was obtained from NCCS, Pune, India and maintained on Dulbecco's Modified Eagle's Medium (DMEM) supplemented with streptomycin (100 U/ml), gentamycin (100 μ g/ml), 10% FBS (Sigma-Aldrich) at 37°C and humid environment containing 5% CO₂. For imaging, MCF-7 cells were cultured on 18 mm glass coverslips in 12 well plates at 2×10^4 cells/well and allowed to grow for 48 hours (till 70-80% confluence). Experiments to assess Cr³⁺ uptake were performed in the same media supplemented with different concentrations of Cr(ClO₄)₃.6H₂O (5, 20, 100, 200, 400 μ M) for 2 hours. Cells were washed twice with phosphate buffered saline before incubating with 5 μ M of **1** in PBS for 20 min at 25°C. The cells were again washed twice with PBS before imaging. Confocal imaging of MCF-7 cells was achieved using NIKON AIR confocal laser scanning microscope using diode laser with excitation at 405 nm. Imaging was carried out with Plan Apo 40X objective lens.

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Notes and references

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