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SENSITIVE AND SELECTIVITY OF FLUORESCENT CHEMOSENSOR FOR Fe^{3+} IONS USING 3,3'-((4-METHOXYPHENYL)METHYLENE)BIS(4-HYDROXY-2*H*-CHROMEN-2-ONE) AND ITS BIOLOGICAL APPLICATIONS

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ABSTRACT
A bis(coumarinyl) methane-based fluorogenic probe behaves as a highly sensitive and selective fluorescent chemosensor for Fe^{3+} ions. The synthesised fluorogenic compound is characterised by NMR and Mass spectra and was used as a selective "*turn-off*" and naked-eye sensor for Fe^{3+} ions over other cations. The localised frontier molecular orbitals (DFT studies) and the solvent polarity-dependent photoluminescence characteristics strongly validate the presence of intramolecular charge transfer from the methoxy unit to the coumarinyl unit. Upon Fe^{3+} addition, the fluorescence is quenched due to the formation of a square planar complex of the probe with Fe^{3+} at room temperature, even in the presence of other interfering ions such as Cd^{2+} and Hg^{2+}. DFT studies support the resultant Intramolecular charge transfer (ICT). The lowest detection limit for Fe^{3+} ions is as low as 2×10^{-7} M. The confocal laser scanning micrographs of HeLa cells confirm the cell permeability of the probe and its ability to detect Fe^{3+} ions in living cells selectively.
Keywords: Chemosensor, Fe^{3+} ion, "*Turn off*" Fluorescence, Bis-Coumarinyl Methane, Live-Cell Bio-Imaging.
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INTRODUCTION

The development of highly selective chemosensors for the recognition of environmentally and biologically important metal ions such as Cu^{2+}, Cd^{2+}, Fe^{3+}, Hg^{2+}, $Li^{2+,}$ and Zn^{2+} has been a key area of research.[1-6] Consequently, many chemosensors have been developed, offering advantages such as high sensitivity, low cost, ease of detection, and suitability as a diagnostic tool for biological purposes.[7-12] Iron, as the fourth most abundant element in the Earth's crust and the most important transition metal in biological systems, plays a crucial role in many biochemical processes at the cellular level for both human beings and in other biological systems.[13-15] It is essential for the proper functioning of all living cells and acts as a cofactor in many enzymatic reactions related to oxygen metabolism, electron transfer, and the mitochondrial respiratory chain[16], as well as in specialised transport and storage of proteins.[17] The deficiency of Fe^{3+} causes anaemia, liver damage, diabetes, hemochromatosis, Parkinson's disease, and cancer.[18,19] The World Health Organisation estimates that the number of anaemic people worldwide is a staggering two billion and that approximately 50% of the occurrence of anaemia can be attributed to iron deficiency,[20,21] while excess iron could accelerate the production of reactive oxygen species, which will deteriorate lipids, nucleic acids, and proteins.[22-25] Excessive Fe^{3+} in the human body has also been found to be related to an increased incidence of dysfunction of certain organs.[26] Therefore, careful control and monitoring of Fe^{3+} ions is of great importance. The considerable importance of iron in biological and environmental systems has led to a burgeoning interest in recent years in the development of selective techniques for both qualitative and quantitative determination of iron in clinical, medical, environmental applications, and also in different industrial samples. Various analytical techniques such as spectrophotometry,[27-29] inductively coupled plasma mass spectrometry,[30] voltammetry[31-33,] and atomic absorption spectroscopy[34] have been used for iron determination. However, in many instances, several

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other metal ions are found to interfere and hence require complicated pre-treatment procedures and necessitate the use of sophisticated instrumentation.[35] To date, the fluorescent molecules and their derivatives, including dansyl, rhodamine, anthracene, BINOL, and so on, have been reported[36-42] to detect $Fe^{3+,}$ and some of which showed a 'turn-off'[43] fluorescent phenomenon due to the intrinsic paramagnetic properties of Fe^{3+}. In recent years, coumarin-based chemosensors have received increasing attention by virtue of their properties such as biocompatibility, cytotoxicity, and easier modification. Coumarin and its derivatives are widely distributed throughout nature, and many exhibit useful biological activity,[44] as a fungicide (thiabendazole), NSAID drug (meloxicam), and are also biocompatible. Interestingly, the carbonyl group of coumarin can coordinate with various metal ions, and hence, coumarin and its derivatives are biologically important and are also useful intermediates in the synthesis of many heterocyclic compounds. Coumarin derivatives have also attracted attention as some of the most popular fluorophores amenable to novel sensor design. Coumarin-based chemosensors have been extensively used as fluorescent labelling reagents for their large molar extinction coefficient, relatively long excitation and emission wavelengths, and high fluorescent quantum yields. Recently, coumarin derivatives have also been widely utilised as excellent chromogenic and fluorogenic dyes.[45] Our interest in developing chemosensors for biologically important cations, anions, and neutral molecules[46- 48] has prompted us to develop a simple Fe^{3+} sensor. Recently modified β-cyclodextrins are reported for various sensor catalytic applications from our group. These unique aspects prompted us to choose probe 1, which features a bis-coumarin derivative prepared using a novel synthetic route involving a propylenediamine-modified β-cyclodextrin under sonication (scheme-1) as a rational design for the present study of selective sensing of Fe^{3+}.

EXPERIMENTAL

Chemicals

All chemicals used were of analytical grade and used without further purification. P-methoxybenzaldehyde was purchased from Alfa Aesar. All the metal salts (LiCl, $MnCl_2{\cdot}4H_2O$, $CrCl_3{\cdot}6H_2O$, $CoCl_2{\cdot}6H_2O$, $NiCl_2{\cdot}6H_2O$, $CuCl_2{\cdot}2H_2O$, $HgCl_2$, $FeCl_3{\cdot}6H_2O$, $FeCl_2$, $CdCl_2$, $AlCl_3$, $ZnCl_2$, $SnCl_2$, $AgNO_3$,) were purchased from Merck. UV-Vis spectra were recorded on a JASCO-500 spectrophotometer with a quartz cuvette (cell length 1cm). The fluorescence spectra were recorded with an Agilent Cary Eclipse Fluorescence Spectrofluorimeter. ^1H and ^{13}C spectra were recorded on a BRUKER 300MHz spectrometer using $CDCl_3$ as solvent and TMS as the internal standard. ESI-MS analysis was performed in the positive and negative ion modes on a liquid chromatography–ion-trap mass spectrometry instrument (LCQ Fleet, Thermo Fisher Instruments Limited, USA). DFT calculations were performed at the B3LYP/LANL2DZ(d) level by using the Gaussian 09 program.

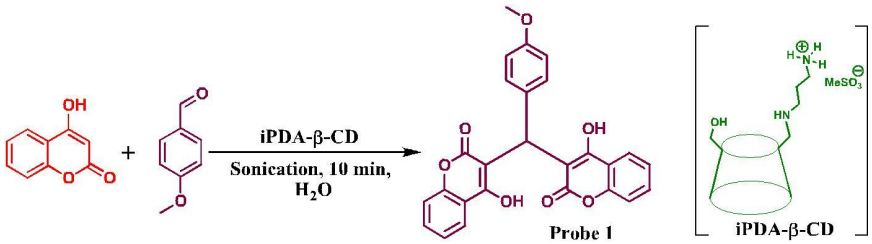
Characterization Details

^1H-NMR (300MHz, $CDCl_3$) δ 7.45–7.34 (m, 4H), 7.38–7.35 (m, 3H), 7.28 (d, J = 1.4 Hz, 1H), 7.15 (d, 2H), 6.94 (d, 2H), 4.81 (s, 1H), 3.78 (s, 3H). ^{13}C-NMR (75 MHz, $CDCl_3$) δ 29.52, 56.97, 104.51, 112.70, 115.29, 116.78, 124.58, 124.99, 130.18, 130.52, 132.35, 153.95, 159.88, 160.63, 164.32. ESI-MS spectrum of Probe 1 $[M + Na]^+$ adduct shows clear molecular weight of the probe, ESI-MS spectrum of Probe 1 + Fe^{3+} [Probe + M + 1].

RESULTS AND DISCUSSION

From β-cyclodextrin, a novel, ionic, propylenediamine-modified β-cyclodextrin (iPDA-β-CD) is prepared. The synthesis of *i*PDA-β-CD was achieved in a single-step, straightforward synthesis with 75% yield. The synthesised *i*PDA-β-CD is soluble in water and exhibits excellent catalytic activity in the synthesis of bis(coumarinyl)methanes. The synthetic pathway to the fluorogenic probe 1 is summarised in Scheme-1. Probe 1 was synthesised in 95% yield, and its structure was confirmed by ^1H-NMR, ^{13}C-NMR and ESI-MS. All data were clearly confirmed to the formation of probe and complex.

The photophysical properties (UV and emission) of the probe 1 as a ligand are studied in detail in the presence of various metal ions, Cd^{2+}, Ag^+, Al^{3+}, Ba^{2+}, Cr^{2+}, Zn^{2+}, Li^{2+}, Co^{2+}, Hg^{2+}, Mn^{2+}, Fe^{2+}, Ni^{2+}, $Cu^{2+,}$ and Fe^{3+} in acetonitrile medium. Only in the case of Fe^{3+} ions, the complexation of probe 1 in acetonitrile is accompanied by a change of colour of the solution from colourless to dark yellow, which is easily observed by the naked eye (Fig.-1).



Scheme-1: Synthetic Pathway for Probe 1

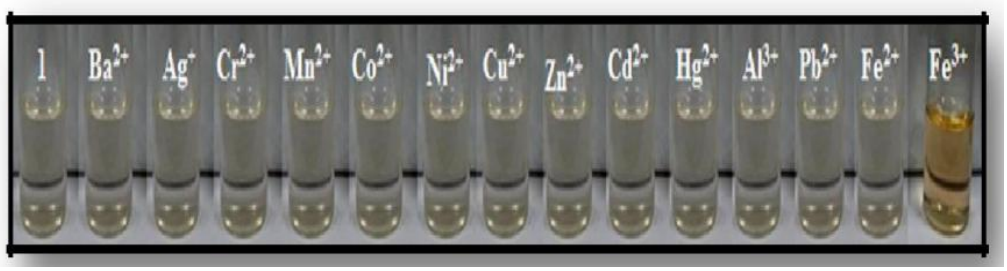


Fig.-1: Colourimetric Response of Probe 1 with 10 equiv. of Various Metal Ions in Visible Light

Addition of five equivalents of other cations as Cd^{2+}, Ag^{+}, Al^{3+}, Ba^{2+}, Cr^{2+}, Zn^{2+}, Li^{2+}, Co^{2+}, Hg^{2+}, Mn^{2+}, Fe^{2+}, Ni^{2+}, Cu^{2+} (Fig.-2a), their chloride salts resulted in no appreciable change in colour, in UV-Vis spectra of the probe 1 in acetonitrile. On addition of all the metal ions to probe 1, the absorption band at 290 nm (shoulder) remained unaffected. However, in the presence of Fe^{3+} ion, the UV-Vis spectrum of probe 1 showed an appreciable change compared to that of the free ligand, indicating ligand-metal interaction. Interestingly, when the Fe^{3+} ion is added, a new absorption band is observed at 500nm, due to the d-d transition of the ligand to the metal. With the gradual addition of Fe^{3+} ion, simultaneous enhancement in intensity of two bands at 350 nm and 500 nm (Fig.-2a) was observed. Further in colourimetric titration, a colour change from colourless to dark yellow was observed upon addition of 0-60μM of Fe^{3+} ions (Fig.-2b).

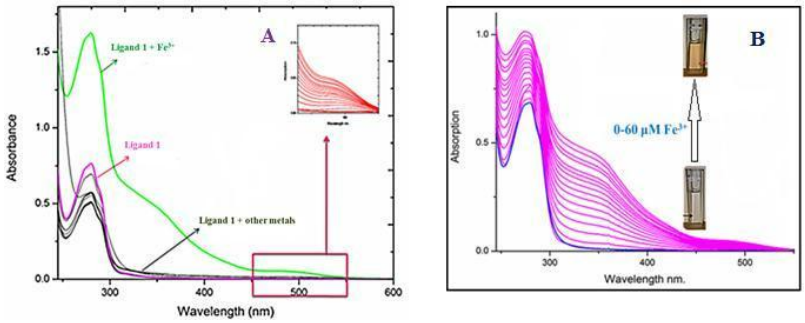


Fig.-2: (A) Absorption Spectra of Probe 1(1×10^{-5} mol L^{-1}), with Addition of Chloride Salts of Cd^{2+}, Ag^{+}, Al^{3+}, Ba^{2+}, Cr^{2+}, Zn^{2+}, Li^{2+}, Co^{2+}, Hg^{2+}, Mn^{2+}, Fe^{2+}, Fe^{3+}, Ni^{2+} and Cu^{2+} (1×10^{-5} mol L^{-1}) in ACN solution; (B) Absorption Spectra of Probe 1 (1×10^{-5} mol L^{-1}), upon Addition of Various Concentration of Fe^{3+} ion (0 to 60 μM) in ACN Solution

The emission spectra of probe 1 in acetonitrile showed no change in fluorescence intensity in the presence of metal ions such as Cd^{2+}, Ag^{+}, Al^{3+}, Ba^{2+}, Cr^{2+}, Zn^{2+}, Li^{2+}, Co^{2+}, Hg^{2+}, Mn^{2+}, Fe^{2+}, Ni^{2+}, Cu^{2+} (Fig.-3a). On the other hand, a substantial fluorescence quenching is observed for probe 1 in the presence of Fe^{3+} ion, and the quenching was weak in the presence of Fe^{2+}. The result indicates that the extent of fluorescence quenching in Fe^{3+} is significantly higher compared to that of Fe^{2+} (Fig.-3a). Further, in fluorometric titration, nearly complete fluorescence quenching was observed on the addition of 0-60 μM equivalent of Fe^{3+} ions (Fig.-3b). The dependence of fluorescence response to the concentration of Fe^{3+} ion in the range of 1 x 10^{-5} to 6 x 10^{-5} mol L^{-1} is given in Fig.-3b. Subsequently, the fluorescence "turn-off" sensing of probe 1 for Fe^{3+} ions has been examined in the presence of other metal ions also. When Fe^{3+} ions were added to various solutions of probe 1 containing different metal ions such as, Cd^{2+}, Ag^{+},

Al^{3+}, Ba^{2+}, Cr^{2+}, Zn^{2+}, Li^{2+}, Co^{2+}, Hg^{2+}, Mn^{2+}, Fe^{2+}, Ni^{2+}, Cu^{2+} in ACN, the binding of Fe3+ ions with probe 1 is not significantly affected by the presence of these other metal ions (Fig.-4).

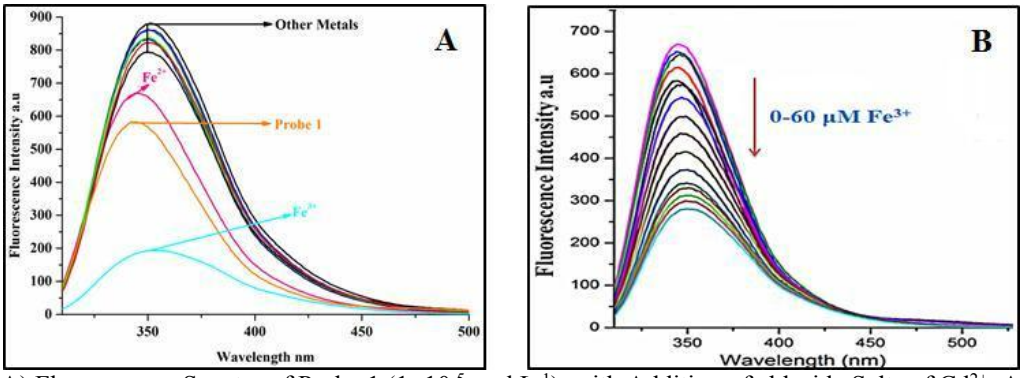


Fig.-3: (A) Fluorescence Spectra of Probe 1 (1×10^{-5} mol L^{-1}), with Addition of chloride Salts of Cd^{2+}, Ag^{+}, Al^{3+}, Ba^{2+}, Cr^{2+}, Zn^{2+}, Li^{2+}, Co^{2+}, Hg^{2+}, Mn^{2+}, Fe^{2+}, Fe^{3+}, Ni^{2+} and Cu^{2+} (1×10^{-5} mol L^{-1}), in ACN Solution with Excitation at 300 nm; (B) Fluorescence Spectra of Probe 1 (1×10^{-5} mol L^{-1}), upon Addition of Various Concentration of Fe^{3+} ion (0 to 60 µM) in ACN Solution with Excitation at 300 nm

Therefore, the intensity of the fluorescence peak of the binding [Probe 1+Fe^{3+}] at 353 nm can be used to monitor the presence of Fe^{3+} ions alone as well as Fe^{3+} ions in the presence of other metal ions. The proposed binding of probe 1 to the Fe^{3+} ion also finds strong support from the observation of an intense peak at m/z. 501. 501,23 m/z in ESI-MS.

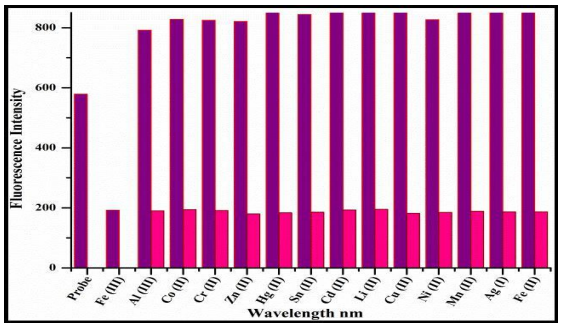


Fig.-4: The Bar Chart Illustrating Fluorescence Response in the Selectivity of Probe 1 (1×10^{-5} mol L^{-1}) for Fe^{3+} ion in the Presence of Other Metal Ions (1×10^{-5} mol L^{-1}). The Lavender Bars Represent the Fluorescence Intensity of Probe 1 in the Presence of One Equivalent of the Other Metal Ions. The Pink Bars Represent the Change in Fluorescence Intensity that Occurs Upon Subsequent Addition of One Equivalent of Fe^{3+} to the Solution containing probe 1 and the Other Metal Ions in Aqueous Solution.

Studies of pH Variation

The fluorescence spectra for probe 1 are also recorded at different pH values. Interestingly, the emission spectra at pH 8 undergo a red shift from 350 nm to 423 nm (Fig.-5a). This change may be due to the formation of enolate anion in the coumarin system (Fig.-5b). Figure-5b represents the ionisation of the phenolic hydroxyl group in the coumarin system and the subsequent resonance stabilisation of the enolate anion.

Theoretical DFT Calculations

The potential geometries of probe 1 with Fe3+ ion were optimised using DFT-B3LYP 6-311G and LANL2DZ (d) levels, respectively, using the Gaussian 09 package. The optimised geometry of probe 1, given in Fig.-7, showed effective binding sites to form a 1:1 complex with the Fe^{3}+ ion. The plots and HOMO/LUMO energy levels of probe 1 in the presence and absence of Fe^{3+}, Al^{3+}, Ni^{2+}, and Ag^{+} ions are calculated. Figure-8a shows that in free probe 1, the methoxy group behaves as a HOMO, whereas the coumarin moiety behaves as a LUMO.

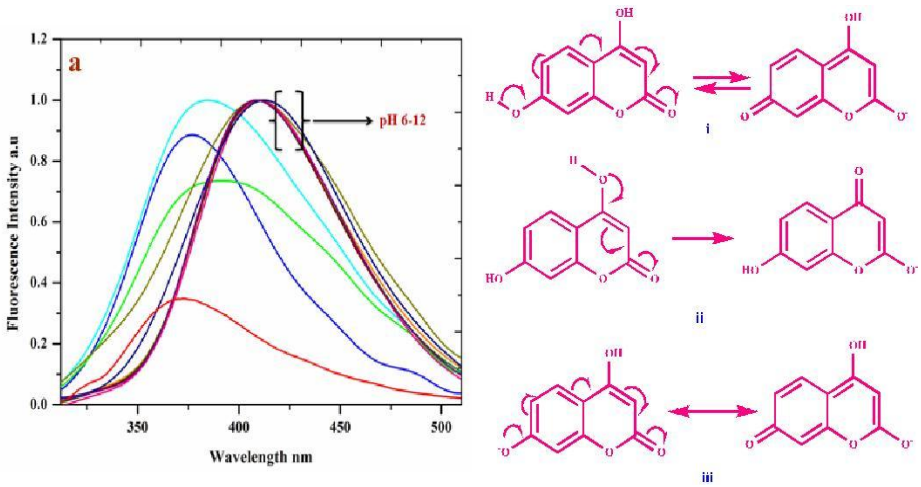


Fig.-5 (a) Fluorescence Spectra of Probe 1 (1×10^{-5} mol L^{-1}), with Various pH Solutions, in Phosphate Buffer (10 mM), pH 8. (λ_{exc} – 300nm, λ_{em} – 425nm, slit: 5 nm/5 nm). Keto-Enol Tautomerism in 1 and Resonance Stabilisation of Enolate Ion

The relatively well-separated charge distribution between HOMO and LUMO levels (Fig.-6a) indicates substantial charge transfer from the methoxy group to the whole coumarin moiety with the -OH group when the molecules are excited.

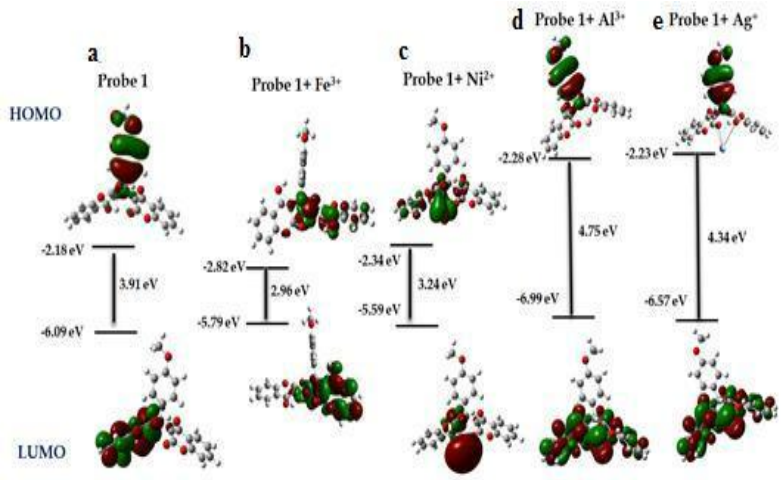


Fig.-6: Frontier Molecular Orbitals Optimized at the B3LYP/LANL2DZ Levels of Theory at Mono and Trivalent

After binding of Fe^{3+} to probe 1, one side of the coumarin moiety has the HOMO character, and the electron transfer to another coumarin moiety, represented as LUMO, is shown in Fig.-6b. In addition, as the ionic radius of Al^{3+} and Ag^{+} are nearly the same as Fe^{3+}, we have also calculated the energy levels in the presence of Al^{3+} and Ag^{+} (Fig.-6d and 6e). The energy levels of divalent Ni^{2+} ions are also calculated (Fig. 7c) to compare with those of Fe^{3+}. Compared to di- and monovalent cations, the bis-coumarinyl moiety binds strongly to trivalent ions. In addition, the observed results demonstrate that the HOMO-LUMO energy gap decreases only with Fe^{3+} ion binding, indicating greater stabilisation, whereas other metal ions of either similar size/charge fail to induce any such decrease in energy gap.

Confocal Cell Imaging

The HeLa cells were incubated with probe 1 (5.0 μM) in HEPES buffer at 36 °C for 30 min and imaged through confocal fluorescence microscopy with 300nm excitation (Fig.-7b). To remove the excess probe 1 present in the extracellular medium, the system was then washed with HEPES buffer five times. HeLa cells incubated with probe 1 was subsequently treated with 10 and 20 μms of $FeCl_3$. In imaging the cells in 10μm $FeCl_3$ solutions, the fluorescence slightly faded (Fig.-7c), and when 20μm of $FeCl_3$ was added,

the fluorescence disappeared further (Fig.-7d). Hence, it can be concluded that probe 1 can also function as a viable chemosensor for $FeCl_3$ ions in living cells.

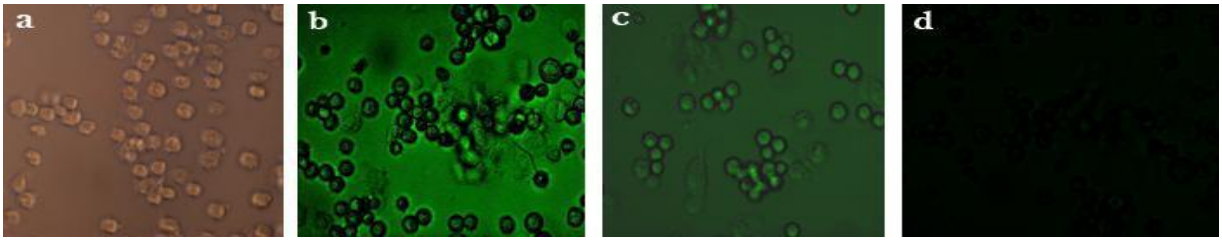


Fig.-7: Bright Field Image and Fluorescence Images of HeLa Cells. (a) Bright-field Image of Probe 1 Treated HeLa Cells (b) Fluorescence Image of HeLa Cells Incubated with Probe 1 (5.0μM) for 4 h at 36°C (c) Fluorescence Image of HeLa Cells Incubated With Probe 1 (5.0 μM) for 30 min and Subsequently Treated with (10.0 μM) $FeCl_3$ for 4 h at 36°C (d) Fluorescence Image of HeLa Cells Incubated with Probe 1 (5.0 μM) for 30 min and Subsequently Treated with (20.0 μM) $FeCl_3$ for 4 h at 36°C

CONCLUSION

In summary, bis(coumarinyl)methane (Probe1) is synthesised and used as a highly selective colourimetric and fluorometric chemosensor for Fe^{3+} ion, based on Intramolecular charge transfer (ICT) mechanism. The probe 1 forms a 1:1 complex with the Fe^{3+} ion, as evident from Job's plot and ESI-MS spectra. The binding mode proposed between Probe 1 and the Fe^{3+} ion, and the fluorescence behaviour, is rationalised from TD-DFT calculations. This sensor system shows a detection limit as low as 2×10^{-7} mol L^{-1}. The confocal laser scanning micrographs of HeLa cells confirm the cell permeability of the probe and its ability to selectively detect Fe^{3+} ions in living cells, and also a full paper was performed to apply to Sustainable Development Goals number 3, 7 and 13.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

The present work is related to *SDG-4: Good Health and Well Being*. All the authors contributed significantly to this manuscript, participated in reviewing/editing and approved the final draft for publication. The research profile of the authors can be verified from their ORCID ids, given below:

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