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RESEARCH ARTICLE

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Phenothiazine–rhodamine-based colorimetric and fluorogenic 'turn-on' sensor for Zn^{2+} and bioimaging studies in live cells

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Abstract

A phenothiazine–rhodamine (PTRH) fluorescent dyad was synthesized and its ability to selectively sense Zn^{2+} ions in solution and in *in vitro* cell lines was tested using various techniques. When compared with other competing metal ions, the PTRH probe showed the high selectivity for Zn^{2+} ions that was supported by electronic and emission spectral analyses. The emission band at 528 nm for the PTRH probe indicated the ring closed form of PTRH, as for Zn^{2+} ion binding to PTRH, the λ_{em} get shift to 608 nm was accompanied by a pale yellow to pink colour (under visible light) and green to pinkish red fluorescence emission (under UV light) due to ring opening of the spirolactam moiety in the PTRH ligand. Spectral overlap of the donor emission band and the absorption band of the ring opened form of the acceptor moiety contributed towards the fluorescence resonance energy transfer ON mechanism for Zn^{2+} ion detection. The PTRH sensor had the lowest detection limit for Zn^{2+}, found to be 2.89×10^{-8} M. The sensor also demonstrated good sensing application with minimum toxicity for *in vitro* analyses using HeLa cells.

KEYWORDS

Fluorescent chemosensor, FRET, Phenothiazine, Rhodamine, Zinc ion

1 | INTRODUCTION

Zinc is the most inexhaustible element on the Earth's crust, and plays a very important role in several physiological and pathological processes such as apoptosis, catalytic function of protein, gene transcription, and enzyme regulation.[1–7] Conversely, zinc deficiency leads to many diseases such as infection susceptibility, sickle cell disease, diarrhoea, diabetes, chronic renal disease, and chronic liver disease.[8–10] Excess accumulated levels of zinc arrest copper and iron absorption in humans and lead to a series of health problems.[11] Therefore, a more accurate and sensitive fluorescence sensor is required to detect Zn^{2+} ions which interference from Cd^{2+} ions.[12–14]

Due to increased levels of Zn^{2+} in water systems, several analytical techniques such as atomic absorption spectroscopy (AAS), atomic emission spectroscopy (AES), inductively coupled plasma mass spectrometry (ICP-MS), X-ray fluorescence (XRF), and electrochemical methods have been used to monitor Zn^{2+} ion levels,[15–19] however these types of techniques are highly time consuming and expensive. Therefore, the development of a new method for Zn^{2+} ion detection

Abbreviations used: $CDCl_3$, deuterated chloroform; DFT, density functional theory; DMSO, dimethyl sulfoxide; EDAX, energy dispersive X-ray spectroscopy; EET, excitation energy transfer (also known as FRET); ESI-MS, electrospray ionization mass spectrometry; FBS, fetal bovine serum; FRET, fluorescence resonance energy transfer; FT-IR, Fourier transform infrared spectroscopy; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; HOMO, higher occupied molecular orbital; LOD, limit of detection; LUMO, lower unoccupied molecular orbital; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NMR, nuclear magnetic resonance spectroscopy; PTRH, phenothiazine–rhodamine-based sensor [(10-hexyl-10h-phenothiazin-3-yl)methylene amino-3′,6′-bis(diethylamino)-2,3-dihydrospiro(isoindole-1,9′-xanthene)-3-one]; PTRH+Zn^{2+}, phenothiazine–rhodamine–zinc complex; SEM, scanning electron microscopy; UV, ultraviolet

in environmental and biological systems is a critical need. In recent studies, processes using fluorescence chemosensors were found to deliver the best analytical techniques for monitoring metal ions in solution, due to their effective features such as simplicity, high sensitivity, ease of function, quick response to fluorogenic and chromogenic colorimetric changes, and biocompatibility in cell imaging, plus generating inexpensive detection systems that delivered considerable advantages over other methods.[20–23]

In recent years, several fluorescent probes for Zn^{2+} ion have been developed, some of which, for example, were turn-on rhodamine-based fluorescent sensors containing bis-triazolyl moieties for Zn^{2+} detection among various other metal ions and that had a detection limit of 1 M for application in *in vitro* HeLa cell imaging.[24] A fluorescent, colorimetric sensor based on 8-aminoquinoline for Zn^{2+} detection in living cells through internal charge transfer (ICT) and chelation-enhanced fluorescence mechanisms has been described previously.[25] A pyrenyl appended hexahomotrioxacalix[3]arene was found to exhibit high selectivity towards Zn^{2+} in mixed aqueous medium[26] and using two simple compounds based on conjugated Schiff bases from indole-derived *p*-phenylenediamine for Zn^{2+} ions.[27]

Here, we describe a phenothiazine-linked rhodamine-based fluorescent sensor, (10-hexyl-10H-phenothiazin-3-yl)methyleneamino-3′,6′-bis(diethylamino)2,3dihydrospiro(isoindole-1,9′-xanthene)-3-one (PTRH), for the selective sensing of Zn^{2+} in water:acetonitrile (8:2, v/v) through fluorescence resonance energy transfer (FRET) (also called excitation energy transfer, EET). This sensor exhibited a 'turn-on' fluorescence sensing behaviour during continuous addition of Zn^{2+} ions. Zn^{2+} ion binding brought about spirolactam ring opening of the receptor unit due to the EET from phenothiazine to the rhodamine moiety. The sensing process involved a red shift in absorbance and a fluorescence spectrum with fluorescence emission from a green to pinkish red colour. The PTRH sensor was used to image Zn^{2+} ions in live cells, with good biocompatibility.

2 | EXPERIMENTAL

All reagents and solvents were purchased from Central Drug House Ltd, India; phenothiazine and rhodamine were obtained from Sigma-Aldrich Co Ltd, India; and metal chloride salts were purchased from Merck & Co., India.

All samples for characterization were prepared at room temperature. FT-IR spectra were recorded using a JASCO-460 plus model spectrometer. ^{1}H-NMR (300 MHz) and ^{13}C-NMR (100 MHz) spectra were obtained on Bruker Avance spectrometer. ESI-Mass spectra were analyzed using an LCQ Fleet mass spectrometer. Absorption and fluorescence measurements were recorded on an AN-UV-7000 spectrophotometer and a JASCO FP-8200 spectrofluorometer, respectively. Surface morphology was imaged using a Vega3 Tescan model scanning electron microscope. EDAX analysis was carried out using a Bruker Nano GMBH model energy dispersive X-ray analyzer. Fluorescent bioimaging was undertaken in living cells and visualized using a Nikon fluorescence microscope.

2.1 | Synthesis

2.1.1 | General procedure for synthesis of 10-hexyl-10H-phenothiazine (1) and 10-hexyl-10H-phenothiazine-3-carbaldehyde (2)

The precursor compounds **1** and **2** were synthesized in accordance with previously reported methods (see Supporting information).[28,29]

2.1.2 | Synthesis of PTRH

Rhodamine B hydrazine (1.28 mmol) and compound **2** (1.28 mmol) were mixed with ethanol (30 ml) and acetic acid (3–5 drops) under a nitrogen atmosphere and the reaction mixture was refluxed overnight. After the reaction was completed, the precipitate was filtered, washed with methanol and dried in a vacuum. Finally, the crude product was purified by column chromatography using ethyl acetate:hexane (2:8, v/v) to furnish pure compounds of the PTRH sensor as a greenish yellow powder. Yield: 81%. Melting point: 180°C. FT-IR (cm^{-1}): 3056 (=C–H, str), 2965–2862 (C–H, str), 1719 (C=O, str), 1461 (C–H, bend), 1306–1114 (C–N, str), 1233 (C–O, str). ^{1}H-NMR: (300 MHz, $CDCl_3$), δ (ppm) 8.22 (s, 1H), 7.98 (d, 1H), 7.45 (t, 1H), 7.40–7.37 (d, 1H), 7.11 (m, 2H), 6.87 (m, 4H), 6.81 (t, 1H), 6.73 (t, 1H), 6.43 (d, 1H), 6.23 (d, 2H), 6.21 (d, 2H), 3.80–3.75 (t, 2H), 3.35–3.28 (q, 8H), 2.71 (s, 1H), 2.26–2.14 (d, 2H), 1.73 (m, 6H), 1.19–1.14 (t, 12H), 0.85 (t, 3H). ^{13}C-NMR: (100 MHz, $CDCl_3$), δ (ppm) 164.92, 152.97, 152.04, 148.92, 133.22, 129.11, 128.19, 127.94, 127.19, 126.72, 123.71, 123.31, 115.39, 114.90, 108.01, 105.96, 97.90, 65.87, 44.33, 31.42, 26.56, 22.57, 13.98, 12.65. Mass (ESI-MS): $C_{47}H_{51}N_5O_2S$ [M + H^+]: 749.48.

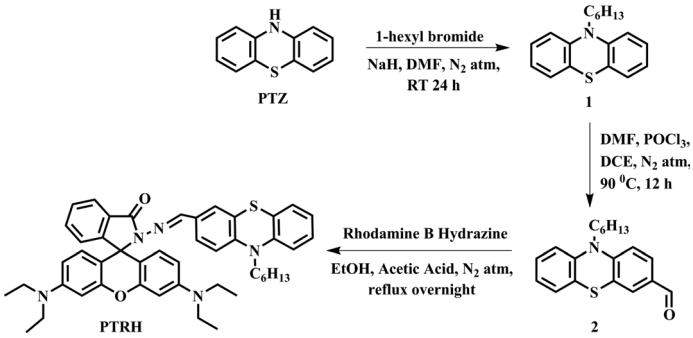
3 | RESULTS AND DISCUSSION

3.1 | Synthesis of PTRH

The PTRH sensor was successfully synthesized through condensation of rhodamine B hydrazine and compound **2** with drops of acetic acid in ethanol (Scheme 1). The purity of the ligand PTRH was established from FT-IR, ^{1}H-NMR, ^{13}C-NMR, and ESI-iass (see Supporting information Figures S1–S4).

3.2 | UV–visible spectral response to metal ions

Influence of various heavy metal ions such as Hg^{2+}, Mg^{2+}, Co^{2+}, Cu^{2+}, Ba^{2+}, Al^{3+}, Mn^{2+}, Pb^{2+}, Cd^{2+}, Fe^{2+}, Fe^{3+}, Ni^{2+}, Ca^{2+}, Na^+, and K^+ (10 μM) on the absorbance of the PTRH ligand (10 μM) was investigated using an electronic spectral method (Figure 1). The spectrum of the free PTRH ligand showed four absorption peaks at 235 nm, 276 nm, 308 nm, and 390 nm. None of the metal ions presumably had made any changes in the absorbance of the ligand PTRH, except that addition of Zn^{2+} ions produced the formation of new band at 559 nm, i.e. a red shift that demonstrated strong n–π* transition. This red shift occurred due to spirolactam ring opening of the rhodamine



SCHEME 1 Synthesis of PTRH

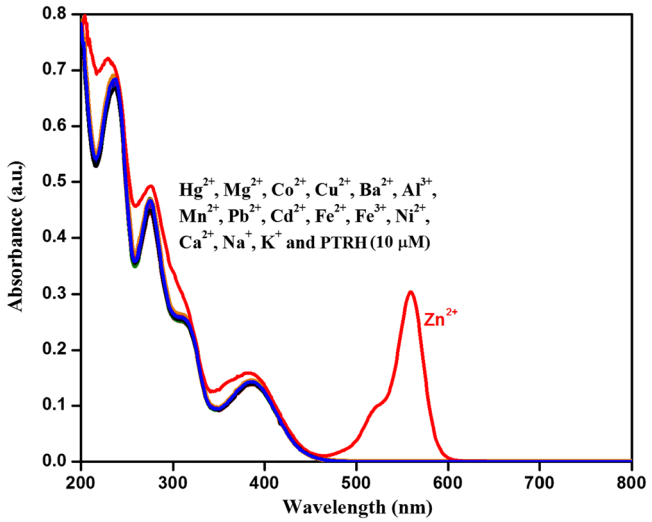


FIGURE 1 UV–visible spectrum of PTRH (10 μM) in the presence of various competitive metal ions in a water:acetonitrile (8:2, v/v) mixture

moiety, induced by energy transfer from the donor (phenothiazine) in the excitation state that was absorbed by the acceptor (rhodamine) unit at the ground state. This excitation resulted in excitation energy transfer that drove the sensing process. Therefore, the electronic spectrum revealed selectivity of PTRH for Zn^{2+} ions.

Energy transformation of the PTRH probe (10 μM) in the presence of Zn^{2+} ions (10 μM) was studied using UV–visible titration studies with varying concentration of Zn^{2+} ions (0–2 equiv.) in a water:acetonitrile (8:2, v/v) system. Figure S5 showed that, on increasing the concentration of the Zn^{2+} ions, ligand absorbance intensity was enhanced linearly and indicated the sensitivity of the probe towards Zn^{2+} ions. This was also observed by the naked eye through colour change from pale yellow to pink under visible light, as shown in Figures S6 and S7.[30,31]

3.3 | Fluorescence titration and selectivity

Fluorescence changes of the PTRH probe (10 μM) on addition of different metal ions (10 μM) were investigated. Compared with observations from absorbance studies, Zn^{2+} alone contributed a major influence on ligand fluorescence. As seen from the spectrum (Figure S8), the free PTRH probe displayed fluorescence at 528 nm following excitation at λ_{em} 559 nm, the emission at 528 nm was characteristic of phenothiazine units. On addition of the Zn^{2+} ion solution, a new intense emission band at 608 nm was observed with simultaneous quenching of the phenothiazine peak at 528 nm. The new band at 608 nm represented the spirolactam ring-opening effect of rhodamine due to binding with Zn^{2+} ion, thereby extending conjugation of the system and a green to pinkish red emission transformation (Figure S9). Fluorescence changes in PTRH following addition of other metal ions was not significantly discriminative; this finding showed the selective nature of the PTRH probe for Zn^{2+} ions in solution.[32,33]

Fluorescence titration of the PTRH ligand (10 μM) with Zn^{2+} ions (0–1.1 equiv.) was performed. The titration plot (Figure 2) indicated that fluorescence of the phenothiazine moiety was gradually quenched at 528 nm with simultaneous enhancement at 608 nm that indicated the spirolactam ring-opening process and increasing conjugation characteristics of the ligand. A clear isoemissive point was observed around 575 nm that reflected the point of equilibrium, i.e. the existence of the two forms of the ligand (the closed ring and the opened ring forms of the lactam unit). Increase in intensity at 608 nm on addition of the Zn^{2+} ion solution was due Zn^{2+} ion binding brought about by formation of a conjugative bridge between the donor and acceptor that favoured EET. A large volume of unstable energy from the donor (phenothiazine) in the excited state was released to excite the receptor (rhodamine moiety) from its ground

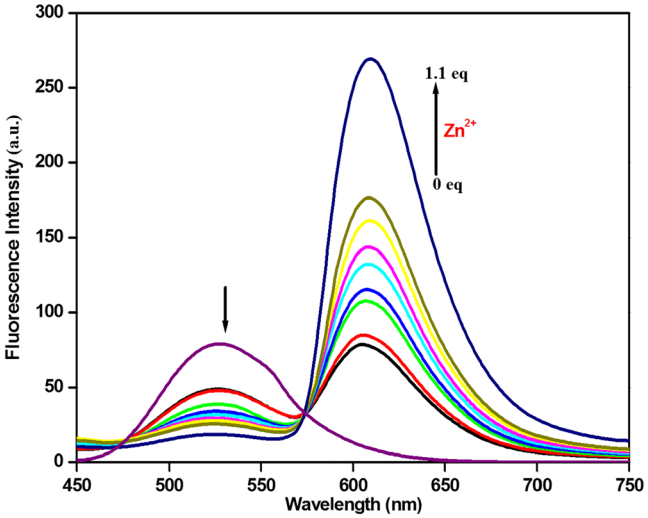


FIGURE 2 Concentration-dependent fluorescence enhancement of PTRH (10 μM) on the addition of various amounts of Zn^{2+} (0–1.1 equiv.) in a water:acetonitrile (8:2, v/v) mixture (λ_{ex} = 559 nm)

state. This energy transfer was notable from the titration spectrum in Figure 2, showing a regular and spontaneous increase in emission from the rhodamine moiety and decrease in phenothiazine emission. Therefore, the FRET process was highly amenable based on direct evidence from the large spectral overlap of the donor (phenothiazine) emission and acceptor (ring opened rhodamine unit) absorption band depicted in Figure 3. Emission analysis also demonstrated that the PTRH ligand was highly selective and sensitive towards Zn^{2+} ions (Figure S9).[34,35]

The stoichiometric binding ratio was established using the continuous variation method (Job's plot). Figure S10 shows the variation in fluorescence with increasing mole fractions of Zn^{2+} ions to PTRH ligand. The figure shows a maximum at 0.5 mole fraction of Zn^{2+} ions that indicated the probability of a 1:1 stoichiometry of PTRH to Zn^{2+}. This result was also in agreement with the ESI-MS peak of the complex at 813.3125 m/z (Figure S11).[36] The association constant of ligand–metal was calculated from fluorescence titration data using the Benesi–Hildebrand equation, and was found to be 1.26×10^6 M^{-1}. A plausible binding mode for PTRH with Zn^{2+} is shown in Scheme 2.

The lowest detection limit of PTRH for Zn^{2+} ion was obtained from a linear fit by plotting fluorescence intensity against the titration concentration (Figure S12). Using the slope of the linear fit, LOD was calculated from the expression LOD = K × σ/S, and found to be 2.89×10^{-8} M, where K = 3.[37]

To investigate the effect of pH on fluorescence, an emission spectrum of PTRH with Zn^{2+} ion at different pH, ranging from 1 to 14, was generated at λ_{em} = 608 nm (Figure S13). Fluorescence intensity of PTRH in the presence of Zn^{2+} did not show any significant change between pH 5 and 14; this result indicated that the spirolactam ring of the rhodamine moiety was in its closed form. When pH was adjusted to between 1 and 5, the fluorescence intensity of the PTRH was reputedly enhanced due to spirolactam ring opening in the amide form of rhodamine. These results clearly indicated that the PTRH sensor was insensitive to pH ranging from 5 to 14 and may work under approximately physiological conditions with very low background fluorescence.[38,39]

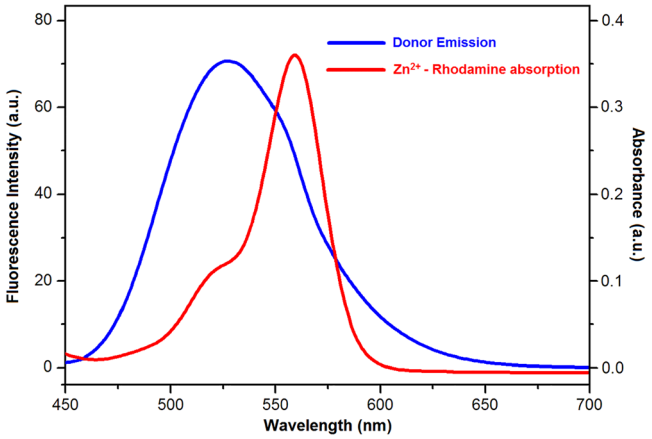


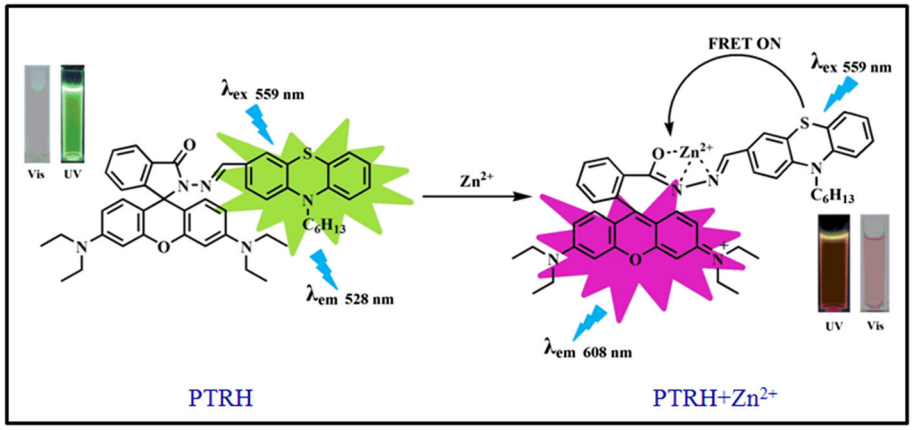
FIGURE 3 Spectral overlap between donor emission and absorption of Zn^{2+}–rhodamine

3.4 | FT-IR titration analysis

To confirm the nature of the complex between PTRH and Zn^{2+}, a Fourier transform infrared (FT-IR) spectrum for PTRH was recorded in the absence and presence of Zn^{2+} ions (Figure 4). The characteristic amide carbonyl (C=O) stretching vibrations at 1719 cm^{-1} shifted to 1684 cm^{-1} in the presence of Zn^{2+}, indicating that the amide spirocarbonyl O atom (CO) of the rhodamine B unit was involved in recognition of Zn^{2+}. A shift in the carbonyl absorption band in the infrared (IR) region proved to be an effective way to confirm this structural change;[40,41] this finding was also consistent with that of previous studies. These results strongly supported the involvement of oxygen atoms in the spirolactam amide in Zn^{2+} ion binding.

3.5 | Proton NMR titration analysis

A ^{1}H-NMR titration was performed by addition of different concentrations (0.0 equiv., 0.5 equiv., and 1.0 equiv.) of Zn^{2+} with PTRH (1.0 equiv.) in $CDCl_3$ (Figure 5). Upon addition of 0.5 equiv. Zn^{2+}, the imine proton H_g in PTRH shifted downfield from 8.22 ppm to 8.29 ppm.



SCHEME 2 Plausible binding mode of PTRH with Zn^{2+} ions

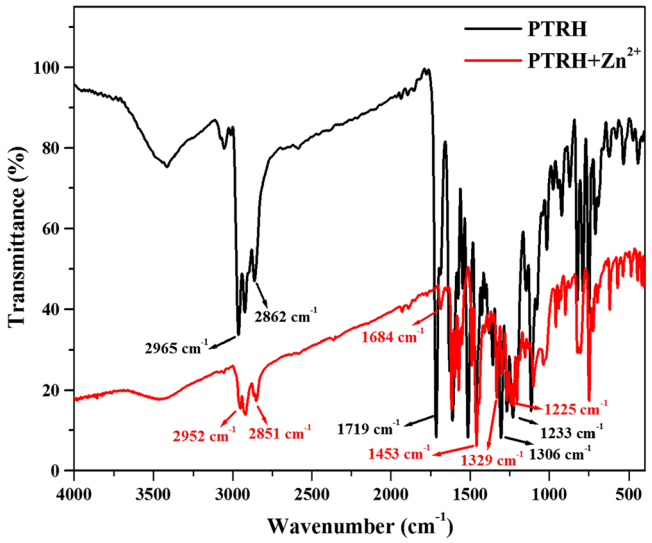


FIGURE 4 Comparison between the FT-IR spectra of PTRH and the PTRH+Zn^{2+} complex

Subsequently, following addition of another 0.5 equiv. Zn^{2+}, the H_g proton further shifted downfield to 8.37 ppm, accompanied by broadened and reduced peak intensities. Protons H_a, H_b, H_c, H_d, H_e and H_f are also shifted downfield upon interaction with Zn^{2+} ions, and indicated that Zn^{2+} underwent ring-opening.[42]

3.6 | SEM and EDAX analysis

Surface morphology of PTRH and PTRH+Zn^{2+} sensors was characterized by SEM .[43] The PTRH sensor exhibited a sandstone-like morphology and the PTRH+Zn^{2+} complex appeared as an agglomerated-like structure, as shown in Figure S14. Changes observed in the PTRH morphology in the PTRH–Zn^{2+} complex indicated the involvement of Zn^{2+} ions in altering the physical properties of PTRH. The EDAX peak at 1.0 kev and 8.6 keV in the PTRH–Zn^{2+} complex, along with C, N, O, S in PTRH, clearly indicated the presence of Zn^{2+} ions in the PTRH–Zn^{2+} complex (Figure S15).[44]

3.7 | Application in living cells

PTRH cytotoxicity was evaluated by adding various concentrations from 0 μM to 20 μM to HeLa cells. More than 98% of the cells remained viable, therefore this sensor could be used for live cell imaging. HeLa cells were incubated with PTRH (20 μM) for 30 min at 37°C, and showed an obvious green fluorescence. After addition of 20 μM Zn^{2+}, cells were incubated for another 30 min, then washed with HEPES buffer three times, following which bright red fluorescence was observed (Figure 6). This effect demonstrated that PTRH was capable of sensing Zn^{2+} ions in living cells.[45]

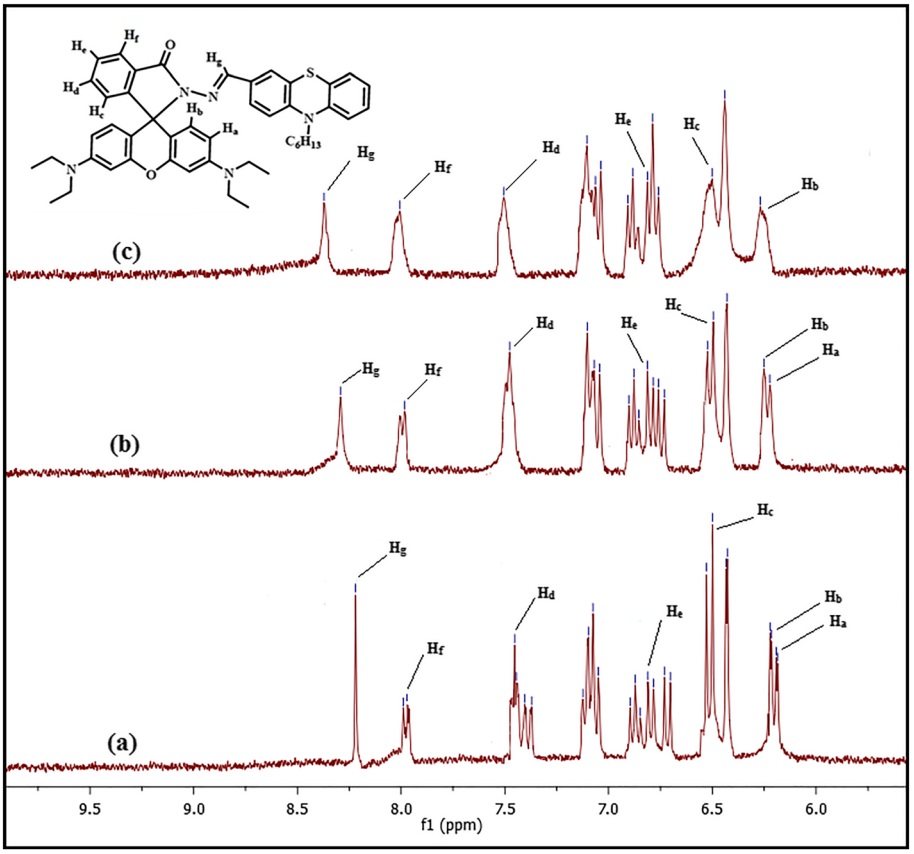


FIGURE 5 Partial ^{1}H-NMR (300 MHz) spectra of: (a) PTRH, (b) PTRH + 0.5 equivalents of Zn^{2+}, and (c) PTRH +1 equivalent of Zn^{2+} in $CDCl_3$

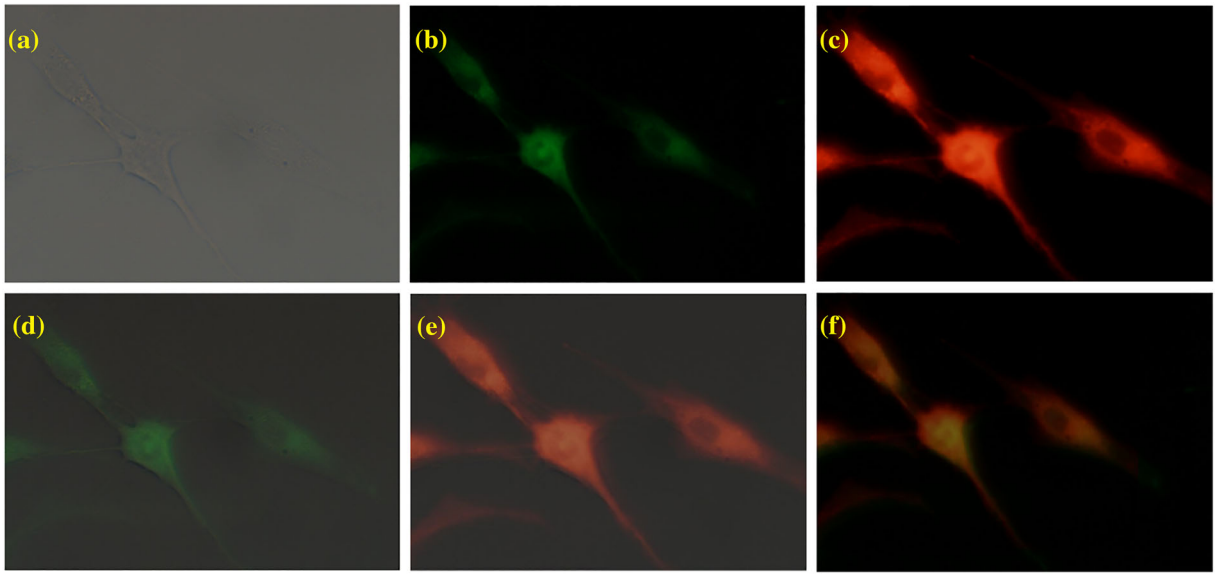


FIGURE 6 Bright field image and fluorescence images of HeLa cells. (a) Bright field image of HeLa cells. (b) Fluorescence imaging of PTRH (20 μM). (c) Fluorescence imaging of PTRH after addition of 20 μM Zn^{2+} ion. (d) Overlap imaging of [a] HeLa cell and [b] PTRH. (e) Overlap imaging of (a) HeLa cell and the (c) PTRH+Zn^{2+} complex. (f) Overlap imaging of (d) and (e)

3.8 | DFT studies

To exploit the knowledge of spatial distributions and orbital energies for the LUMO and HOMO in PTRH and PTRH+Zn^{2+}, DFT calculations were carried out with B3LYP and 6-311G/LANL2DZ basis sets using the Gaussian 09 program (Figure 7). Frontier molecular orbitals in PTRH and the PTRH+Zn^{2+} complex were derived from optimized geometries (shown in Figure S16). In PTRH, the HOMO was expanded on phenothiazine, while the LUMO was centred on the spirocycle of the rhodamine moiety and more restricted. The π electrons in the HOMO of the PTRH+Zn^{2+} complex were primarily positioned in rhodamine, while the LUMO was finely positioned on the guest Zn^{2+} ion. The energy gaps of the LUMO and the HOMO in the probe and the complex were calculated, and found to be PTRH = 3.5244 eV

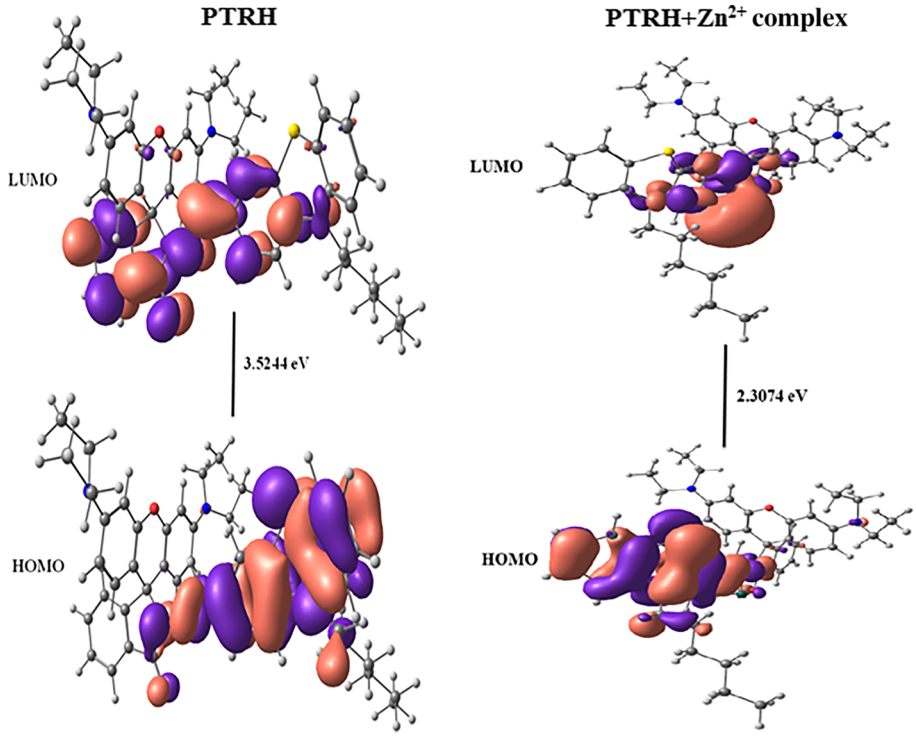


FIGURE 7 Frontier molecular orbitals of PTRH and the PTRH+Zn^{2+} complex obtained from DFT calculations using the Gaussian 09 program

and PTRH+Zn^{2+} = 2.3074 eV, respectively. This result demonstrated that the energy gaps of the LUMO and the HOMO in the Zn^{2+} complex were small when compared with that of the PTRH sensor.[46]

4 | CONCLUSION

We successfully synthesized a new fluorescence sensor, PTRH, composed of phenothiazine (the energy donor) coupled to a rhodamine (the energy acceptor) moiety that showed specific colorimetric, fluorogenic, and ratiometric fluorescence responses towards Zn^{2+} ions through the FRET process. Upon binding to Zn^{2+} ions, the spirolactam ring in rhodamine opened and induced an excitation energy transfer from phenothiazine to rhodamine, evident from the large spectral overlap of the donor emission band and the acceptor absorption band. Selective binding of Zn^{2+} ions was highly spontaneous, with a fluorescence colour change from yellowish green to pink. The PTRH sensor provides a reasonable lowest detection limit over the nanomolar range for Zn^{2+} ions. Nontoxicity, permeability and fluorescence transparency of the sensor make it useful as an imaging agent for detecting Zn^{2+} ions in living cells.

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