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A novel phthalazine based highly selective chromogenic and fluorogenic chemosensor for Co^{2+} in semi-aqueous medium: Application in cancer cell imaging

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Abstract

A new phthalazine based chemosensor **3** was developed for the highly selective and sensitive detection of Co^{2+} in mixed solvent system CH_3CN/H_2O (1:1, v/v). In the presence of Co^{2+}, colour of the solution **3** was changed from yellow to green, the absorption maxima of **3** was red-shifted from 383 nm to 435nm, and the fluorescence of **3** at 550 nm was significantly enhanced. The sensor **3** showed a detection limit down to 25 nM by forming a complex species with Co^{2+} in 1:1 stoichiometry. Furthermore, by means of confocal laser scanning microscopy experiments, it has demonstrated that it can be used as a fluorescent probe for monitoring Co^{2+} in living cells.

Keyword: phthalazine based chemosensor, Co^{2+}, colorimetric, fluorescent enhancement, nanomolar detection limit.

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Introduction

The growth of colorimetric and fluorescent probes for the sensing of environmentally or biologically important metal ions has increased significantly in current years. Fluorescence supported chemosensors for metal ions have stretched great attention due to their analytical aids in various environmental applications in quick and accurate detection of target metal ions up to a very low concentration, and in medicinal field for bioimaging of target analyte in live cells [1-11]. In order to develop of a suitable fluorescent chemosensor, the designing of recognition unit for a target metal ion is very important. With this potential, there is growing curiosities in the chemistry of hydrazine's and hydrazones because of their prospective biological applications. A lot of reports have pointed out that the physically active molecules become more carcinostatic and bacteriostatic upon coordination with the metal ions [12-15].

The discovery of Co^{2+} in the midst of the transition metal ions has paying attention much attention because cobalt is one of the mainly significant trace elements in human beings. In the organization of vitamin B_{12}, this metal obtains fraction in a digit of significant duties in much natural principle [16-18]. Cobalamin is necessary for biological synthesis like DNA, persistence of the nervous system, arrangement of red blood cells, enlargement and development of small kids [19]. Comparable to previous vital parts, cobalt can be a smaller amount lethal than non-fundamental metals like other transition metals [20]. Though, the anxious constancy of Co^{2+} all the way through the required metabolism of living things lift flaw to sickness [21]. The excess of Co^{2} from standard allowable boundary be able to reason harmful effects such as cardiomyopathy, vasodilatation, and flushing while its lack in beings and mammals consequences in anaemia [22]. Since a possible require of extremely responsive diagnostic process, readily available be vast curiosity in the growth of Co^{2+} discriminating probes in the current times used for a variety of biochemical and ecological functions.

Herein, as a part of our on-going research on analytes recognition, we have investigated the cations recognition ability of a novel receptor **3** in semi-aqueous (CH_3CN/H_2O, 1:1) medium by various spectroflourometric and spectrophotometric experiments. All the results indicate that the receptor **3** showed a unique interaction mode for the detection of Co^{2+} with high selectivity and sensitivity over other cations.

Experimental

All solvents and reagents were obtained from commercial grade and were used without further purification. ^{1}H and ^{13}C NMR spectra were recorded on a Varian NMR mercury system 300 spectrometer operating at 300 MHz and 75 MHz respectively in $CDCl_3$. The fluorescence and UV-Visible spectra were recorded on fluoromax-4 spectrofluorometer and Shimadzu UV-2400 in the range of 200-600 nm respectively, at room temperature using 1 cm path length quartz cuvette. IR spectra were obtained in KBr discs on a Chemito FT-IR spectrometer in the 4000-400 cm^{-1} region.

Spectroscopic studies

For all spectroscopic experiments, the solvent ratio between CH_3CN and H_2O was kept as 1:1 (v/v). Solutions of various cations (100 μM) were prepared in double distilled water. A stock solution of receptor **3** (1 mM) was prepared in CH_3CN, and then the working solution of 10 μM was prepared by appropriate dilution with CH_3CN/H_2O (1:1, v/v). In selectivity experiments, the test samples were prepared by placing appropriate amounts of the cations solution into 2 mL of **3** (10 μM). In titration experiments, a quartz optical cell of 1 cm optical path length was filled with a 2 mL solution of **3** (10 μM) and then the incremental amount of metal ions solution was added gradually using a micropipette. All spectral readings were recorded at room temperature and 1 min after the addition of metal ions. For fluorescence measurements, the receptor was excited at 405 nm and the emission scaned from 400 nm to 700 nm.

Synthesis of receptor 3

Receptor **3** was conveniently synthesized via the condensation of 1-(pthalazine-4-yl) hydrazine (**1**) with 2-hydroxy-3-methyl-5-isopropylbenzaldehyde in ethanol (**2**) (Scheme 1). The compound **1** (160 mg, 0.0103 M) was treated with compound **2** (120 mg, 0.01 M) in anhydrous ethanol (20 mL). Then, the mixture was refluxed for 2 hrs. After cooling, the solid was collected and washed with anhydrous ethanol followed by drying to get a yellow solid. (Yield: 69 %), m.p-191^{0}C, ^{1}H-NMR (300 MHz, $CDCl_3$)δ: 1.27-1.29 (6H, d, C-9,10H), 2.47 (3H, s, C-7H), 3.36-3.45 (1H, sept, C-8H), 6.69-6.72 (1H, d, C4-H), 7.13-7.16. (1H, d, C3-H), 7.48.-7.51 (1H, t, 22-H) 7.64-7.67 (2H, m, C20,21-H), 7.99 (1H, s, C17-H), 8.39-8.42 (1H, t, C19-H), 8.99 (1H, s, =C11-H), 10.23 (1H, s, N13-H), 12.17 (1H, s, OH). ^{13}C-NMR (75 MHz, $CDCl_3$): δ 13.29, 120.80, 123.44, 124.46, 126.01, 127.20, 127.30, 131.65, 132.02, 135.87, 138.23, 147.91, 148.67, 156.81, 160.45. LC-MS: m/z, calcd for (M^+) 321.30; Found 321.21. $C_{19}H_{20}N_4$; Calculated (%): C 71.22, H 6.29, N 17.48; Found (%): C 71.67, H 6.33,N 17.30.

Computational details

The structural optimization of **3** and its complex with Co^{2+} was performed by applying the density functional theory (DFT) method at the B3LYP level of theory in the gas phase using the computational code Gaussian 09W [23]. The 6-31G(d,p) basis set was assigned to C, H, O and N atoms. The LANL2DZ basis set with effective core potential was employed for the Co atom.

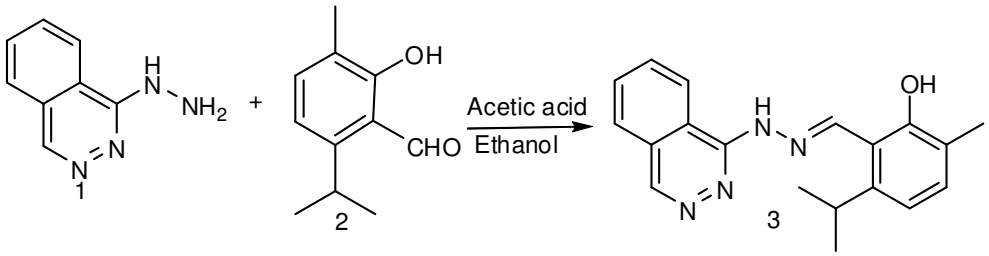
In vitro cell imaging

HeLa cells were procured from NCCS, Pune and grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 1% L-glutamine-penicillin *streptomycin*. The cells were maintained at 37°C in a humidified atmosphere of 5% CO_2. Cells after reaching 80-90% confluence were trypsinized and seeded

on glass coverslips placed in 12-well plate and allowed to adhere for overnight. At the time of experiment, complete media was replaced with serum free medium. The cells were incubated with Co^{2+} (2.5 μM) for an hour. After an hour of incubation with Co^{2+} (2.5 μM), the cells were then incubated with **3** (0.178 μM) for further 50 minutes. The cells were washed twice with Phosphate Buffer Saline (1X PBS) and then fixed with 100% methanol for 5 minutes and further washed with 1X PBS for 10 minutes. The cover slip was then mounted on a glass slide using glycerol and observed under fluorescence microscope (Leica DMI 6000B) using 20X objective under UV filter. The fluorescence images of cells were captured through an attached CCD camera using LAS software.

Results and Discussion

Receptor **3** could be conveniently synthesized via the condensation of 1-(pthalazine-4-yl)hydrazine with 2-hydroxy-3- methyl-5-isopropylbenzaldehyde in ethanol (**Scheme 1**). The molecular structure of **3** was confirmed by ^{1}H-NMR, ^{13}C-NMR, and LC-MS spectroscopic methods (Figure **S1-3**, Supporting information, SI). The chemical shift of CH=N proton was found at 11.56 ppm, probably due to the existence of an intramolecular N···H–O hydrogen-bonding interaction between the phenolic-OH and imine-N atom [24].



Scheme 1. Synthesis of receptor **3**.

Schiff bases bearing imine-N and phenolic-OH are well known chelating agents for transition metal ions. This fact encouraged us to examine the cation recognition ability of **3** towards different metal ions. To assess the binding capacity of receptor **3** towards different metal ions, we carried out UV–Vis and fluorescence experiments of **3** in CH_3CN/H_2O (1.1,

v/v) by adding aliquots of different metal ions (Cr^{3+}, Mn^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, K^{+}, Na^{+}, Mg^{2+}, Cs^{2+}, Cd^{2+}, Al^{3+}, Hg^{2+}, Sr^{2+}, Ca^{2+}, Ba^{2+} and Pb^{2+}) as their nitrate salts. The absorption spectrum of receptor **3** was characterized by the presence of two absorption bands at 286 and 383 nm attributable to the π-π^{*} and n-π^{*} transitions, respectively (**Figure 1**). Upon addition of 0.5 equivalent of Co^{2+} ion in receptor **3** (**Figure 1**), a reasonable red-shift from 383 nm to 435 nm was observed in the absorption spectrum of **3**. However, other metal ions failed to influence the absorption spectrum of **3**. Further, with the addition of incremental doses of Co^{2+} to a solution of **3** (**Figure 2**), the intensity of peak at 383 nm was decreased with the concomitant rise in the absorbance at 435 nm most probably due to the intramolecular charge transfer (ICT) occured between the cation bound phthalazine, imine and hydroxyl groups. Also, three well-defined isosbestic points at 291, 325 and 410 nm were observed, which indicated the formation of only one type of complex species **3**.Co^{2+} in solution.

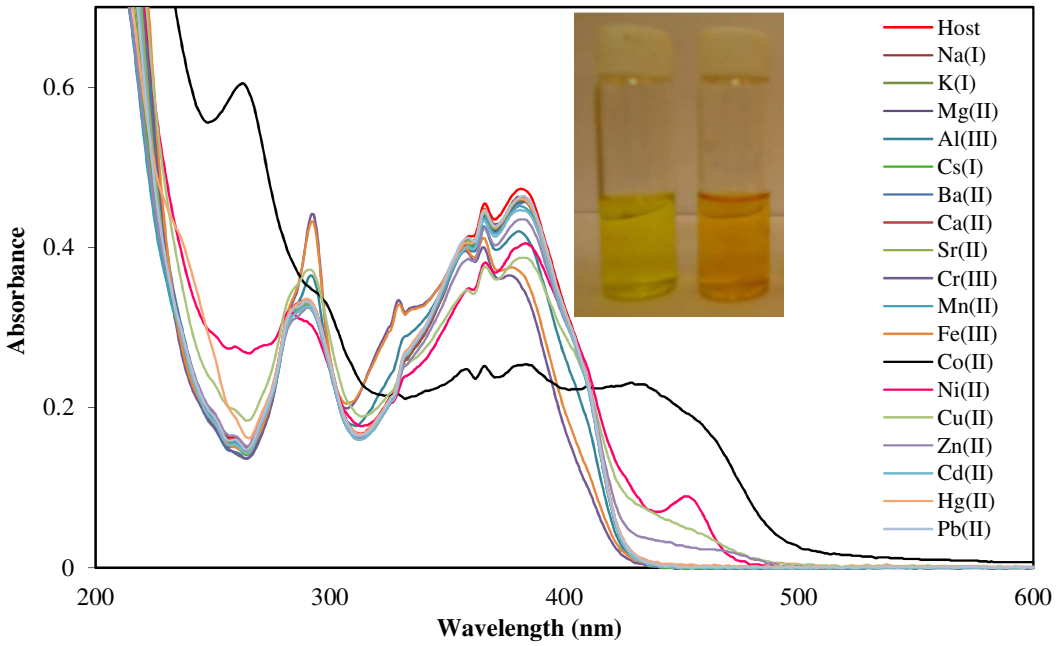


Figure 1. UV-Vis spectra of **3** (10 μM) in the absence and presence of 1.0 equiv. of different metal ions in CH_3CN/H_2O (1:1, *v/v*).Inset figure shows colour change on the addition of Co metal solution (right side only receptor 3 left side receptor 3+ Co).

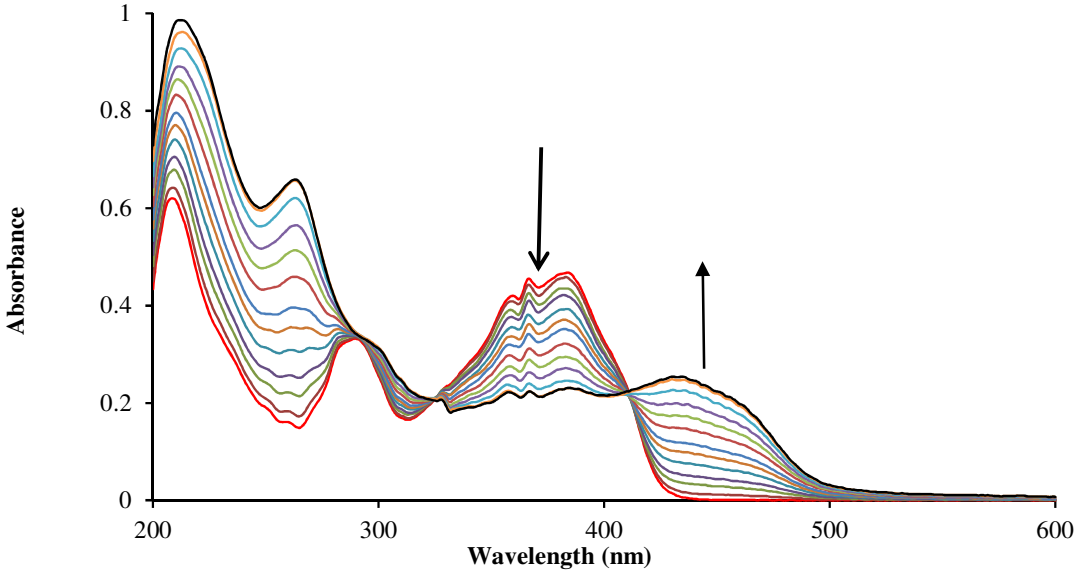


Figure 2. Absorption spectra of **3** (10 μM) upon successive addition of Co^{2+} (100 μM) in CH_3CN/H_2O (1:1, *v/v*).

As illustrated in **Figure S4**, among the tested metal ions, the absorption spectrum of **3** was significantly altered only in the presence of Co^{2+}. Further, to examine the practical applicability of **3** as a Co^{2+} selective chromogenic sensor, competitive experiments were carried out in the presence of Co^{2+} (100 μM) mixed with one equiv. of Cr^{3+}, Mn^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, K^+, Na^+, Mg^{2+}, Cs^{2+}, Cd^{2+}, Al^{3+}, Hg^{2+}, Sr^{2+}, Ca^{2+}, Ba^{2+} and Pb^{2+} ions (1000 μM) (**Figure S5**). No significant difference in the intensity was observed by comparing the profile with and without the presence of other metal ions which clearly delineated the high selectivity and specificity of **3** towards Co^{2+}. In addition, we have also discovered that upon addition of Co^{2+}, the solution of **3** exhibits an obvious yellow to green colour change at the micromolar level without any significant interference from other ions. Therefore,

chemosensor **3** can be developed for the selective 'naked-eye' detection of Co^{2+} in semi-aqueous media.

The nitrate salts of Cr^{3+}, Mn^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, K^{+}, Na^{+}, Mg^{2+}, Cs^{2+}, Cd^{2+}, Al^{3+}, Hg^{2+}, Sr^{2+}, Ca^{2+}, Ba^{2+} and Pb^{2+} ions were next used to evaluate the cation selectivity and binding properties of **3** by fluorescence spectroscopy (**Figure 3**). As expected, **3** exhibits excellent fluorescence enhancement towards Co^{2+} over all other alkali and alkaline earth metal ions, and transition metal ions signifying the application for the highly selective detection of Co^{2+} ion. The enhancement of fluorescence was attributed to the occurrence of the strong complexation of Co^{2+} with **3**, resulting in the inhibition of the C=N isomerisation process which decreased non-radioactive decay of the excited-state [25].

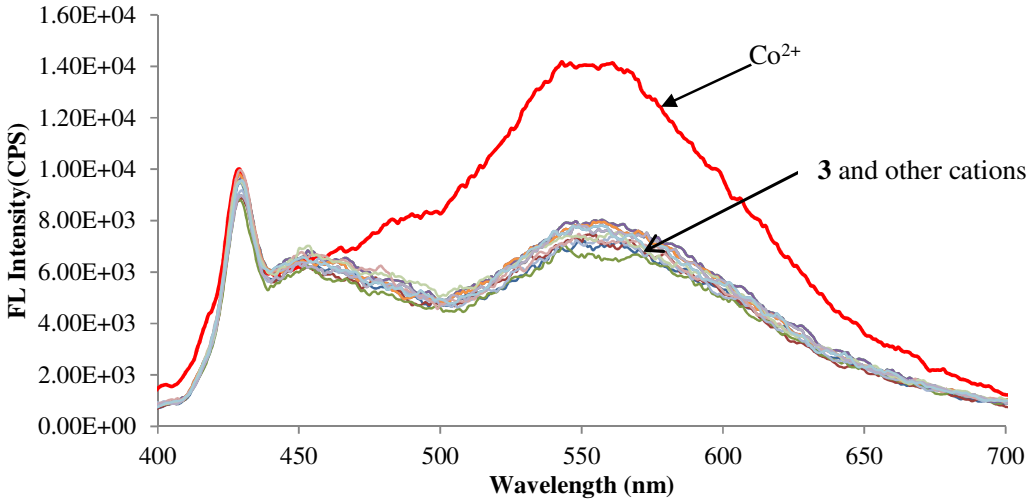


Figure 3. Fluorescence spectra of receptor **3** with addition of different cations (1.0 eq.) as their nitrate salts.

To acquire more information on the sensing mechanism, the fluorescence titration of **3** (10 μM) was carried out by adding increasing amounts of Co^{2+} in CH_3CN/H_2O (1:1, v/v). The receptor **3** showed a weak emission at around 550 nm (λ_{exc} = 405 nm). Upon continuous addition of Co^{2+}, the emission band peaked at 550 nm of **3** was significantly enhanced due to the inhibition of the C=N isomerisation at the excited state that caused a large chelation-

enhanced fluorescence (CHEF) effect (**Figure 4**). The detection limit based on the definition by IUPAC (*C*DL = 3*S*b/*m*) was estimated to be 25 nM (**Figure S6**) which was much lower than the maximum allowable reported detection limit [26, 27].

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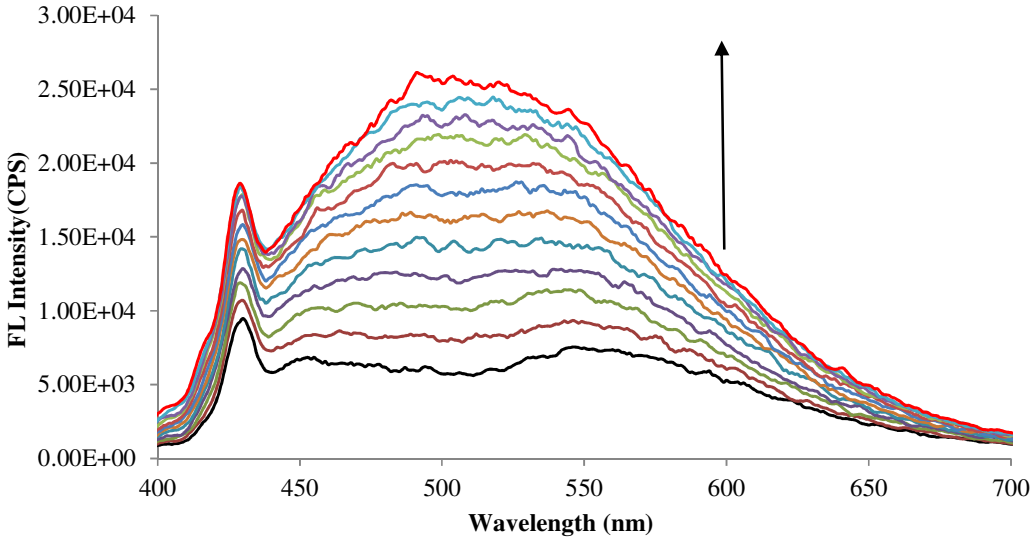

Figure 4. Fluorescence emission spectra of receptor **3** (10 μM) upon successive addition of Co^{2+} (100 μM) in CH_3CN/H_2O (1:1, *v*/*v*), λ_{exc} = 405 nm.

The Job's plot [28] as shown in **Figure 5** indicates that the **3**.Co^{2+} complex was formed in a 1:1 stoichiometry, which was also supported by a good linear fitting of the change in fluorescence intensity against 1/[Co^{2+}] drawn by assuming a 1:1 binding stoichiometry. The association constant of 400000 M^{-1} between **3** and Co^{2+} was determined from the Benesi-Hildebrand Plot (**Figure S7**) [29]. Further, the reversibility of the formation of the **3**.Co^{2+} complex was scrutinized with the addition of EDTA. The fluorescence of **3**.Co^{2+} was reversed and also the green colour was disappeared upon the addition of excess EDTA solution. Also, the consequence of pH on the sensing ability of **3** was experienced. The Co^{2+} sensing ability of **3** was less responsive towards pH.

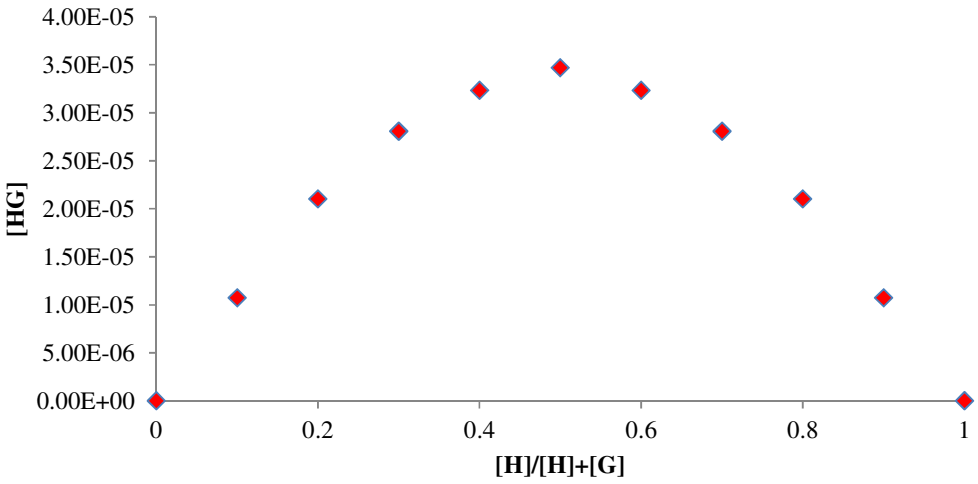

Figure 5. 1:1 Stoichiometry of the host-guest relationship realised from the Job's plot between receptor **3** and Co^{2+} ion.

The structural optimization of **3** and its complex with Co^{2+}, and their electronic properties were obtained at the B3LYP/6-31G(d,p)/LANL2DZ level in the gas phase. The enol-imine form of **3** was found to be more stable than its keto-amine form by 9.63 kcal/mol. Also, the receptor **3** was stabilized with the presence of the intramolecular hydrogen bond between the phenolic-OH and the imine-N of length 1.723 Å (**Figure 6a**). Upon complexation with Co^{2+}, the interaction energy ($E_{int} = E_{complex}-E_{receptor}-E_{Co^{2+}}$) was lowered by -202.48 kcal/mol which indicates the formation of a stable complex (**Figure 6a**). Further, the highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO) of **3** and **3**.Co^{2+} was analyzed. The possible intramolecular charge transfer occured between the receptor **3** and Co^{2+} was identified on drawing the frontier molecular orbitals (FMOs) plots of **3.**Co^{2+} complex (**Figure 6c-d**). The HOMO of **3.**Co^{2+} was mainly distributed over phenolic unit as observed in case of receptor alone. However, the charge distribution of alpha and beta LUMOs of **3.**Co^{2+} were located over Co atom and entire molecule respectively. Further, the ICT process caused the lowering in the band gap between the HOMO and LUMO of **3** on complexation with Co^{2+} which caused the red-shift in the absorption band.

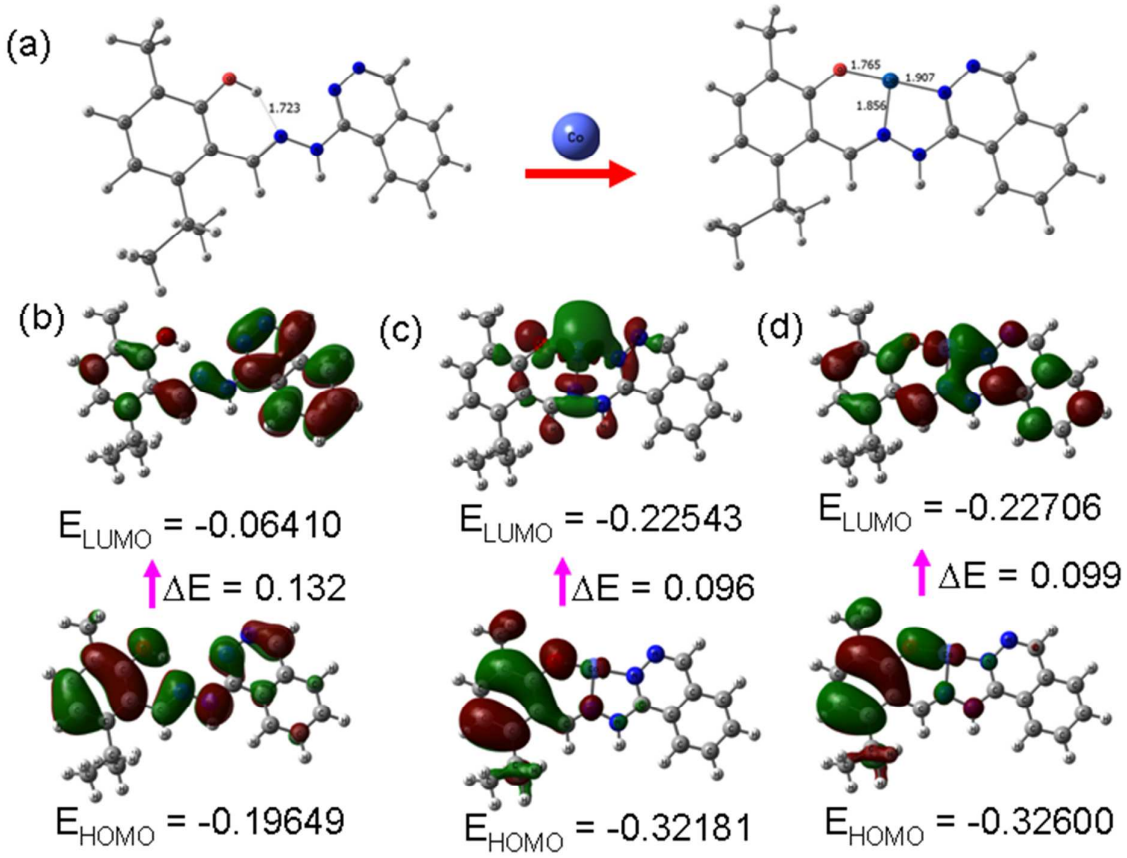

Figure 6. DFT computed (a) optimized structure of **3** and its complex with Co^{2+}, and the HOMO and LUMO diagrams of (b) receptor **3**, (c) alpha and (d) beta MO's of **3**-Co^{2+} complex.

To support the biological utility of the synthetic receptor **3**, it was investigated for sensing Co^{2+} in cells (**Figure 7**). The cells incubated with receptor **3** (0.178μM) alone or in combination with Co^{2+} (2.5μM), fluorescence was observed under UV filter. But no fluorescence was observed in case of the cells incubated only with Co^{2+} (2.5μM). These results demonstrate that receptor **3** and Co^{2+}combination can be used for cellular imaging.

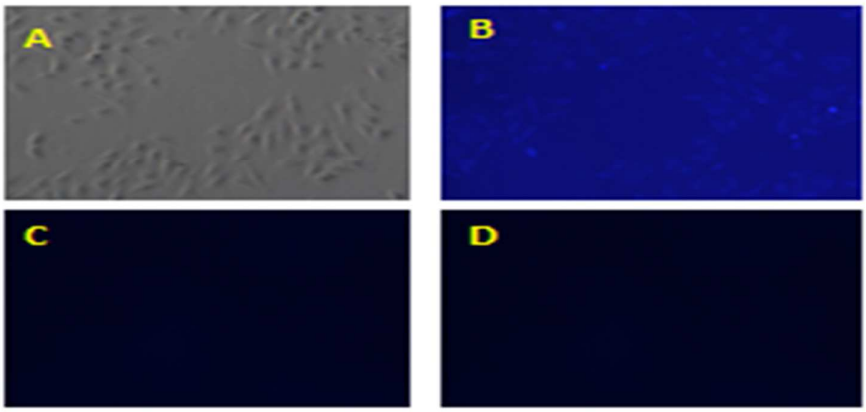

Figure 7 The images were taken in an inverted fluorescence microscope (Leica DMI6000B) under 20X objective. **A**) Phase contrast image of the control cells; **B)** Fluorescence image of the cells treated with both receptor **3** (0.178μM) and Co^{2+} (2.5μM); **C**) Fluorescence image of the control cells under UV filter; **D**) Fluorescence image of the cells treated with Co^{2+} (2.5μM) only.

Conclusion

In summary, we have developed a new easily accessible turn-on fluorescent receptor **3** based on a pthalazine conjugate. The receptor **3** selectively responds to Co^{2+} by chromo- and fluorogenic changes, and also facilitates "naked-eye" detection of Co^{2+}. The background metal ions showed small or no interference with the detection of Co^{2+}. More importantly, this chemosensor showed a nanomolar detection limit of 25 nM. In addition, this probe was successfully applied for imaging intracellular Co^{2+}.

Acknowledgments

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