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A novel fluorescent "turn-on" sensor for the biologically active Zn^{2+} ion

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Abstract

A novel linear Schiff-basedreceptor (**L**) containing two imino phenol units was developed for the selective fluorescent sensing of Zn^{2+} with a discriminating enhancement of 12 folds over the other surveyed cations. Within the huge plethora of Zn^{2+}receptors, this receptor is quite worth to mention because of its simple synthesis, low cost, no interference of Cd^{2+} and the judicious response availed with discernment. The spectroscopic (UV-Vis, fluorescence and mass) results, few mathematical models for determining analytical parameters such as binding constant (K_a), binding stoichiometry, detection limit etc and the frontier molecular orbitalswere analyzedto explore the sensing ability of **L** and the mechanism.

Keywords: Fluorescent 'turn-on' sensor, Zn^{2+}, Schiff base, DFT.

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1. Introduction

Molecular detection is the foundation for understanding many biological functions [1-5]. In the recent years, the growth of artificial receptors for recognizing cationic, neutral, and anionic species are growing because of their important roles in a wide range of biological, environmental, and chemical course of actions [6-10].These receptors are suitably derived using different light-emitting groups to develop chemosensors which can be applied for the qualitative and quantitative detection of the target species.Chemosensors based on colorimetric and fluorescent responses with possible applications in biological corridor have received immense interest because of many advantageous features like simplicity, high sensitivity and selectivity, easy of detection and tunability [11-14].

The transition metal ions play an important role inmany fundamental physiological processes in organisms ranging frombacteria to mammals [15]. It is therefore significant to understand their environmental advantages and risks to the living systems. Zinc is a ubiquitous and indispensable element having second highestabundance after iron in the humanbody,which playsmultiple roles in both extra- and intra-cellular functions [16]. The total concentration of Zn^{2+} in different cells varies from nano to millimolar range.The excessiveconcentration of Zn^{2+} above cellular needs causestoxicity and also displaces other essential metalions that act as co-factors in enzyme-catalyzed reactions [17].Therefore, the development of fluorescent chemosensors to selectively detect and monitor intracellular Zn^{2+} ions is certainly an important issuein the recent years.In this context, numerous efforts have been devoted for the development of such chemosensors displaying phenomena like quenching, enhancement or ratiometric responseswith the help of different signalling mechanisms such as photoinduced electron transfer (PET), C=N bond isomerisation, excited-state intramolecular proton transfer (ESIPT) [18].

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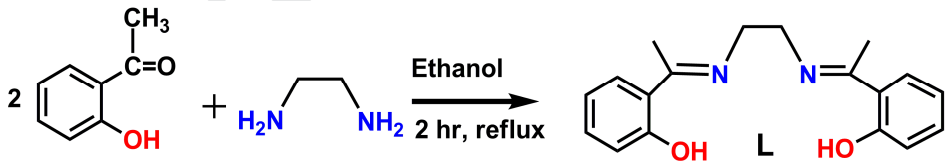
2-Hydroxyacetophenone is a popular substrate for nucleophilic addition reactions by virtue of its carbonyl group which can be activated by the neighbouring phenolic hydroxyl group of the same molecule through intramolecular hydrogen-bonding [19]. Based on this concept, chemosensors with 2-hydroxyacetophenone functionality as the binding site have been developed for the selective detection of different cationsand anions [20]. In this paper, we have designed and developed a Schiff base receptor using 2-hydroxyacetophenone scaffold for the fluorometric determination of Zn^{2+} by fluorescence 'turn-on' response induced by the inhibition of PET process and/or C=N isomerization.

2.Results and Discussion

The receptor **L** was synthesised by adopting the reported method [21] by Schiff base condensation reaction of the corresponding 1-(2-hydroxyphenyl)ethanonewith 1,2-diaminoethanein ethanolic medium (**Scheme 1**). The ^{1}H NMR spectra of receptor **L** displaysa significant downfield signal at 15.94 ppm for the phenolic protons, indicating the presence of intramolecular hydrogen bonding between the phenolic-OH and the neighbouring imine-N atom (**Figure S1-3**).



Scheme 1. Synthesis of receptor **L.**

The ability of **L** to complex differentcations was explored with the absorption and fluorescence spectrophotometer in mixed MeCN/H_2O (99:1, *v/v*) solution, use of this solvent mixture has solved the solubility problem of ligand and allowed it to be used as a chemosensor. Among all the tested cations (Cr^{3+}, Mn^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Pb^{2+}, Cd^{2+}, Bi^{2+}) as their nitrate salts, only Zn^{2+} was found to cause obvious spectral changes in the absorption spectra of **L** (**Fig. S4**) and no particular changes were observed in the presence of

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other cations. The free receptor **L** exhibited two absorption peaks with maxima at254 nm and 320 nm which were attributed to π→π* and n→π* transitions associated with the azomethine chromophore and the imine nitrogen atom in conjugation with the phenol group respectively.Also, the existence of receptor **L** predominantly in the enolimine form can be depicted from the absence of absorption band above 350 nm. On selective interaction of Zn^{2+} with the imine-N and phenolate ions of **L**, the absorption bands of **L** were appreciably red-shifted due to the intramolecular charge transfer (ICT) process which brings the lowest excited state closer to the highest ground state. The absorption spectral titration of **L** with the successive addition of Zn^{2+} was shown in Fig. 1. With the addition of Zn^{2+}, the receptor bands at 254nm and 320nm were attenuated while new bands appeared at longer wavelength 274nm and 358 nm (Δλ = 20 nm and 38 nm respectively). The spectral titrations exhibited four isosbestic points at 241 nm, 260 nm, 296 nm and 333 nm which clearly indicate the formation of a well- defined **L**.Zn^{2+} complex and result was further supported by the addition of 0 to 300 µL of zinc nitrate solution that produces a noticeable colour change.

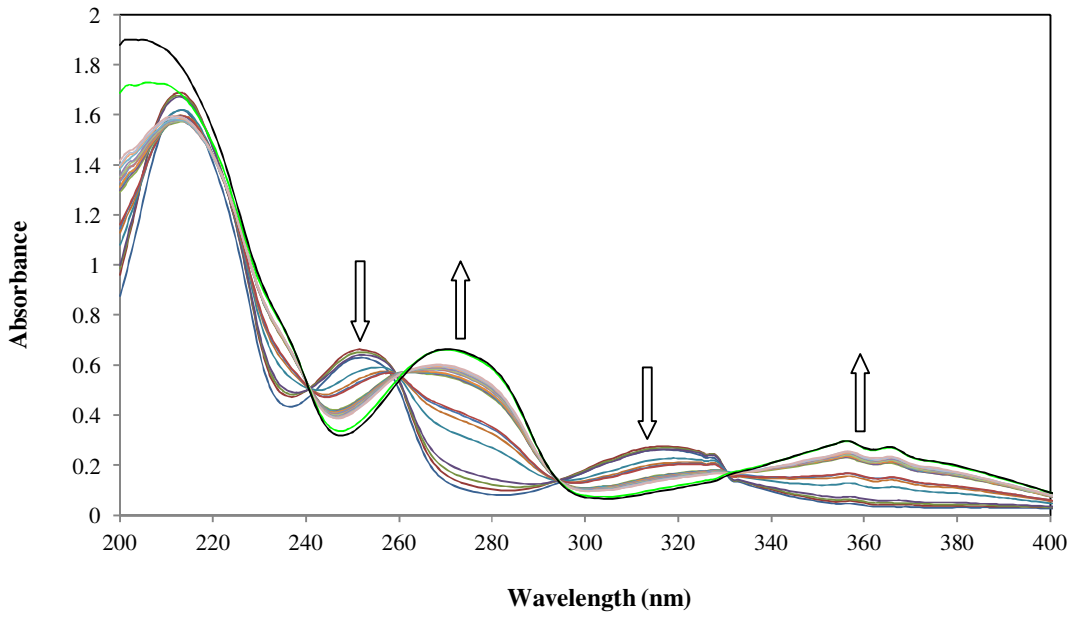

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Fig. 1. Spectrophotometric titration of **L** (0.1mM, 2000 µL) upon successive additionof Zn^{2+} (1mM, 0-300 µL) in MeCN/H_2O (99:1, *v/v*).

The DFT computed molecular structure of **L** and its **L**-Zn^{2+} complex was shown in Fig. 2. The optimized structure of **L**indicates the presence of intramolecular hydrogen bonds of length 1.628 Å. Also, the enolimine (OH) form of **L** was found to be energetically more stable than the ketoenamine (NH) formby -8.62 kcal/mol.The optimized structure of **L**-Zn^{2+} complex (Fig. 2) produce a distorted tetrahedral geometry with the average Zn-N and Zn-O bond lengths of 2.096 Å and 1.936 Å respectively. The band gap between the highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO) of **L** was lowered on complexation with Zn^{2+} which caused the red-shift in the absorption band of **L** (Fig. 3). Furtheranalysis of frontier molecular orbitals (FMOs) plots of **L** and its **L**-Zn^{2+} complex indicate the intramolecular charge transfer occurred between the two iminophenol units on complexation with Zn^{2+}.

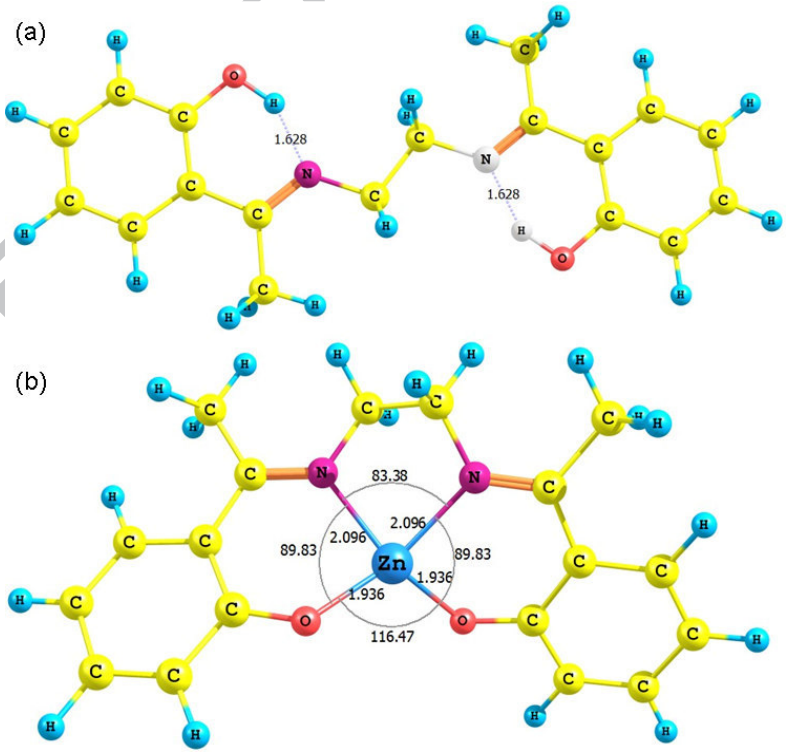

Fig. 2. Optimized structure of receptor (a) L and its (b) L.Zn^{2+} complex.

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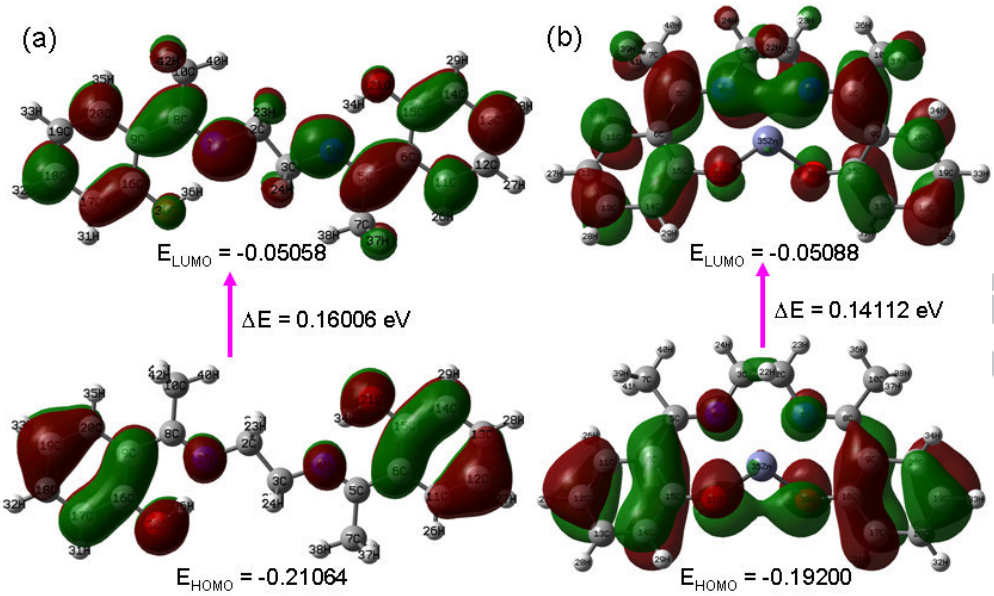

Fig. 3. The DFT computed HOMO and LUMO diagram of (a) **L** and (b) its **L**-Zn^{2+} complex.

The fluorescence properties of **L** was studied upon addition of 0.5 equiv. of Cr^{3+}, Mn^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Pb^{2+}, Cd^{2+}, Bi^{2+} in MeCN/H_2O (99:1, *v/v*) solution (Fig.4). The receptor **L** showed weak fluorescence emission at ~500 nm upon excitation at 320 nm presumably due to the PET from the imine-N to the fluorophore and/or the C=N bonds isomerization in the excited states. Upon addition of Zn^{2+}, prominent fluorescence enhancement was observed at 450 nm with the ratiometric fluorescence responses ($\Delta\lambda$ = 50 nm, anti-strokes or blue shift) (**Figure S5**). In contrast, no appreciable change in the emission behaviour of **L** was observedwith other tested metal ions including Cd^{2+}. The suitable coordination geometry conformation of the chelating Schiff-based receptor **L**and the selective binding of Zn^{2+} close to fluorophore may have influenced the imine and hydroxyl moieties. Consequently, a large chelation enhanced fluorescence (CHEF) effect was observed upon complexation with Zn^{2+} which inhibited the PET process from the electron-donating group to the fluorophore and/or the C=N isomerisation [22]. Further, the fluorescence response (Fig.5)

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of **L** indicates the unique selectivity for the $Zn^{2+}$with 12 folds enhancement among the other tested metal ions.

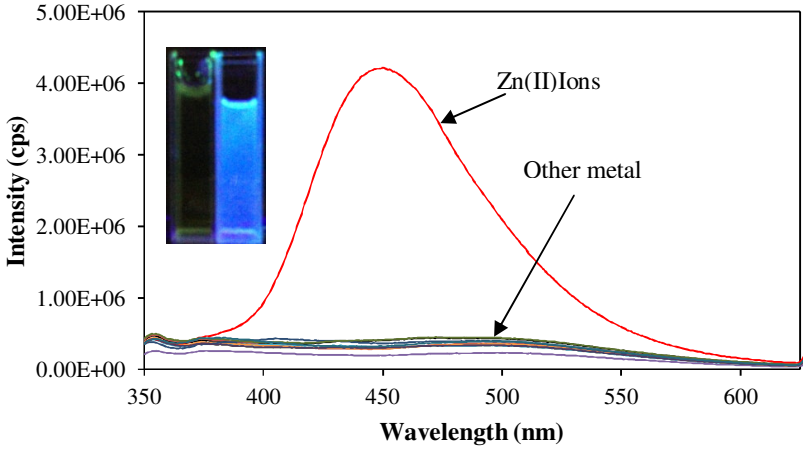

Fig. 4. Fluorescence spectra of **L** (0.1 mM) upon addition of 100 µL of respective metal ions (1.0mM).

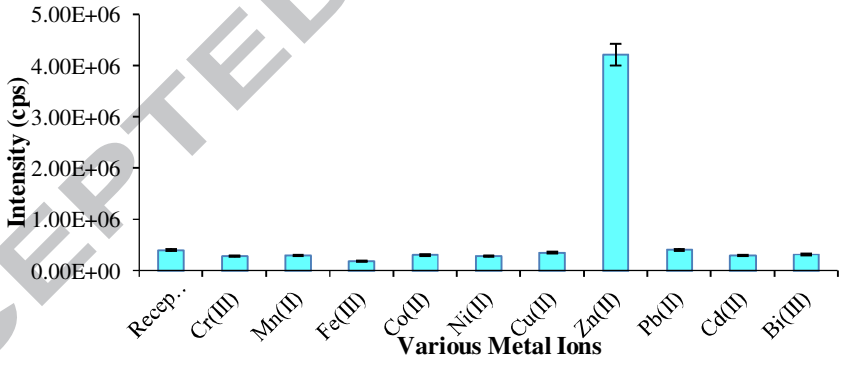

Fig. 5. Fluorescence response of **L** (0.1mM) in MeCN/H_2O (99:1, *v/v*) uponaddition of different metal ions (100µM, 0.5 equiv.).

The practical applicability of **L**as selective fluorescence sensor for Zn^{2+} was investigated by performing a competitive experiment for the estimation of Zn^{2+} (1 equiv.) in the presence of other cations such as Cr^{3+}, Mn^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Pb^{2+}, Cd^{2+}, Bi^{2+}(3 equiv.). No significant difference in the fluorescence responses of **L** for Zn^{2+} was

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observed by comparing the intensity with the co-existence of other metal ions (Fig. 6). The unique binding for Zn^{2+} suggests an interesting view to develop receptor **L**as a fluorosensor for Zn^{2+}even in the presence of other metal ions including Cd^{2+}.

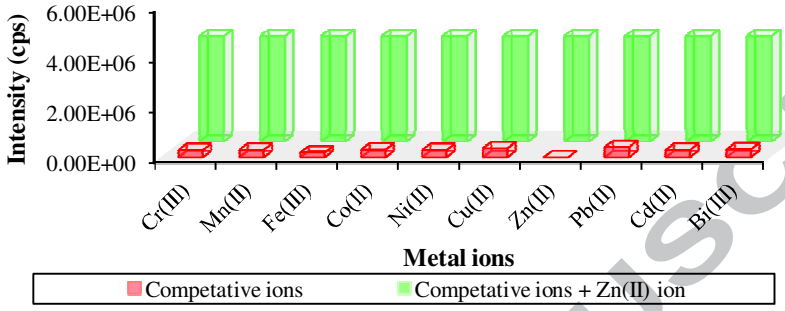

Fig. 6. Interference of respective metals in Zn^{2+} ion detection with receptor**L**.

Fluorometric titration for **L** was next carried out by successive addition of Zn^{2+} to obtain the analytical parameters such as binding constant and detection limit (Fig. 7). The fluorescent intensity increases continuously at 450 nm accompanied by a blue shift of 50 nm in the emission profile of **L**. According to the IUPAC definition, the limit of detection (DL = $3S_b/m$, *S_b: standard deviation of blank, m: slope of the regression line*) was 16 µM [23], which can be obtained from the linear range of the regression curve covering concentration of Zn^{2+} from 5-100 µM (Fig. 8). The lower detection limit is of importance for the practical use of the targeted receptor **L** as selective and significant sensor for Zn^{2+} ions.

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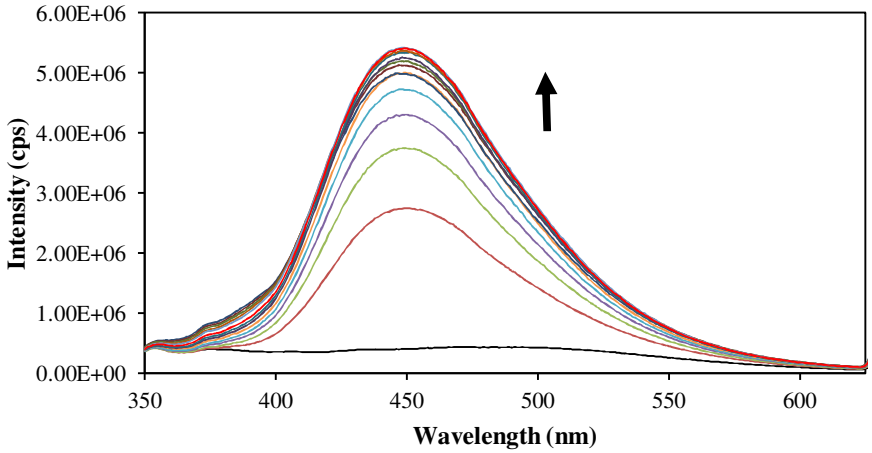

Fig. 7. Fluorescence spectra of **L** (0.1 mM) upon continuous addition of increasing amount of Zn^{2+} (0–200 μL) in MeCN/H_2O (99:1, *v/v*) solution (λ_{ex} = 320 nm).

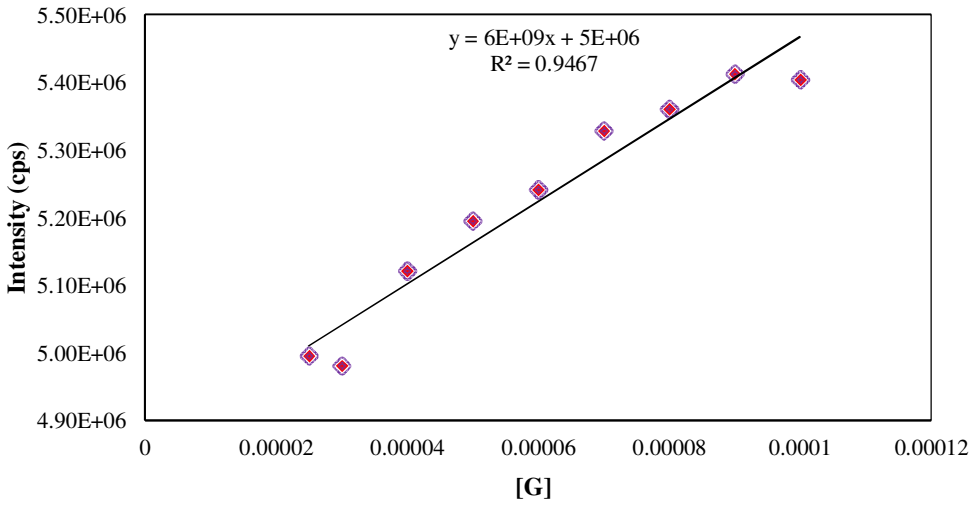

Fig. 8. Normalized fitting for determining the limit of detection.

The binding stoichiometry between receptor**L** and Zn^{2+} was determined by the Job's continuous variation method (Fig. 9) [24]. The results indicate the formation of a 1:1 complex which was also confirmed by the normalized plot (**Figure S6**). The binding constant (K_a) for the **L**.Zn^{2+} complex was calculated from the fluorescence titration experiments (Fig. 7) by mathematical models such as Benesi–Hildebrand, Scatchard and Connor's linear fitting methodologies [25] and was found to be about $(1.72 \pm 0.28) \times 10^5$ M^{-1} (Fig. 10-12).

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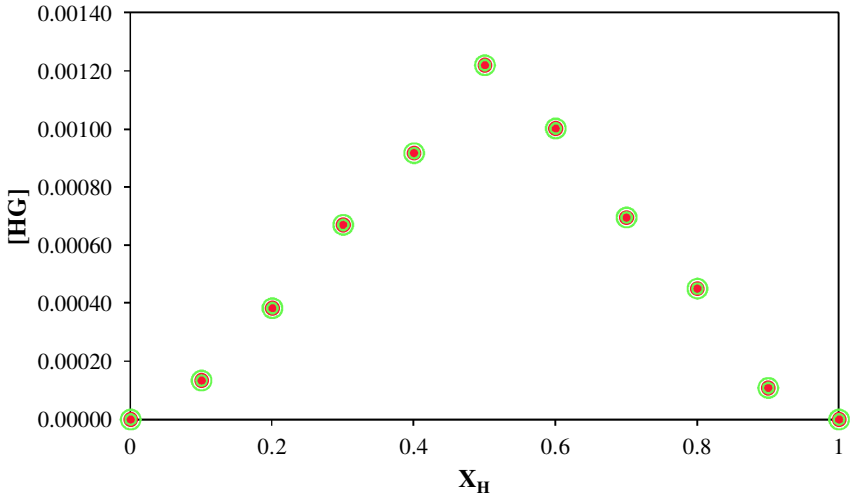

Fig. 9. Job's plot between **L** and Zn^{2+}, wherethe concentration of [HG] was calculated as [HG] = ΔF/Fo×[H]

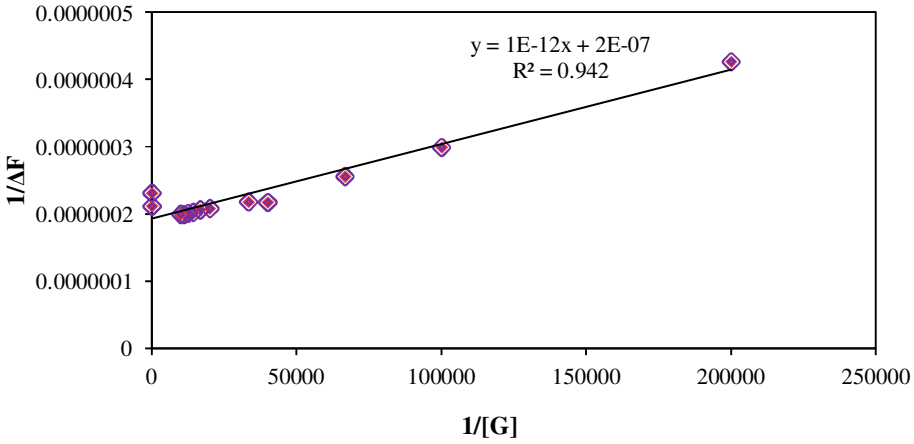

Fig. 10. Benesi-Hildebrand Plot of 1/ΔF versus 1/[G] , *Ka* = Intercept/slope = 2.00 × 10^5 M^{-1}.

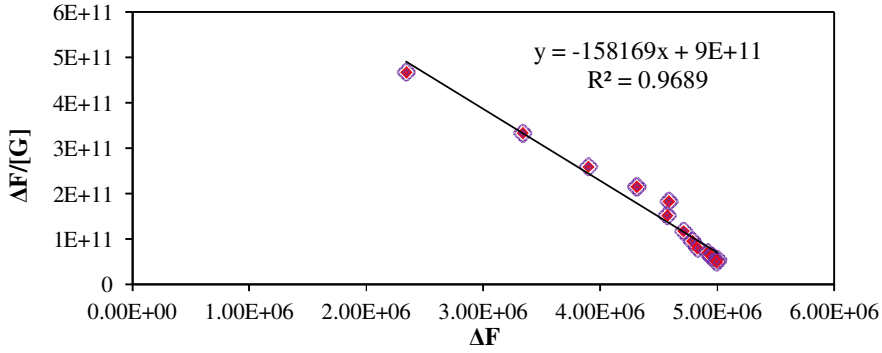

Fig. 11. Scatchard Plot of 1/ΔF versus 1/[G] , *Ka* = slope = 1. 58 × 10^5 M^{-1}.

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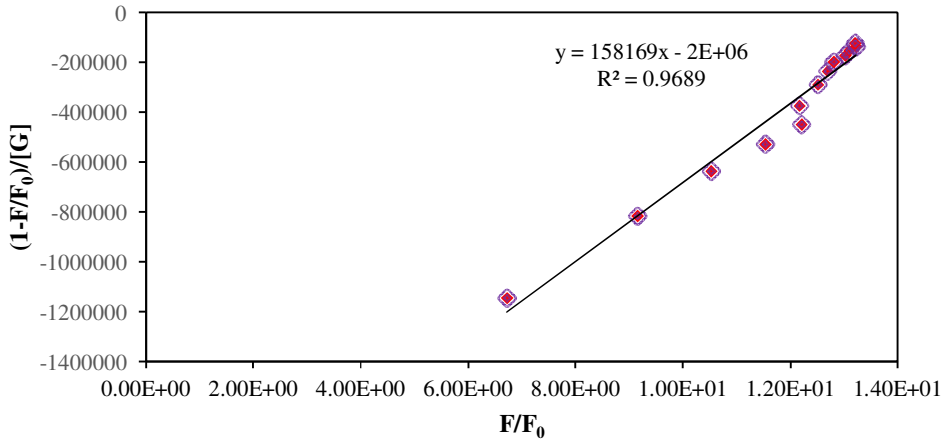

Fig. 12. Connor's fitting Plot of 1/ΔF versus 1/[G] , *Ka* = slope = 1. 58 × 10^5 M^{-1}.

The recognition behaviour of **L**with $Zn^{2+}$was further confirmed by FT-IR spectroscopy, which gives important sign of differentiation between the spectra of receptor **L** and its complexwith Zn^{2+} in the region of 4000–450 cm^{-1} (**Figure S7**). In the IR spectra, the stretching frequency of $\nu_{C=N}$for the free receptor **L** appeared at 1606 cm^{-1} but when the receptor **L**coordinated with Zn^{2+}, alone pair transfer occurred from C=N to Zn^{2+} which induces extreme shift in the IR peak of $\nu_{C=N}$ to 1609 cm^{-1}[26]. It also confirmed the involvement of nitrogen atom of **L** in the complex formation. Next, the IR of free receptor **L** showed a broad band at 3396 cm^{-1} which can be assigned for ν_{OH} group. On complexationwith Zn^{2+}, deprotonation of OH group took place which resulted in the disappearance of ν_{OH} band. Further, the MALDI/TOF–MS data confirmed the formation of 1:1 complex between the receptor**L** and Zn^{2+} ion [(MS (ESI): m/z (Calculated) $C_{18}H_{18}ZnN_2O_2.SO_4$: 456.70, found 456.78](**Figure S8**).

To justify the specific and sensitive response of **L** for Zn^{2+}, **L** was utilized for *in vitro* cell imaging assay. A very faint fluorescence was observed from the cells under UV filter when the cells were incubated with **L** (0.011μM) only for 2 hours where as no fluorescence was observed in the cell when incubated with Zn^{2+} (25μM) only. However, an enhancement

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in the intensity was observed when the cells were incubated using both receptor **L** (0.011μM) and Zn^{2+} (25μM) simultaneously under same experimental condition (**Figure13**). Therefore, these results demonstrate that **L** can be used for the detection of Zn^{2+} in biological samples like cells.

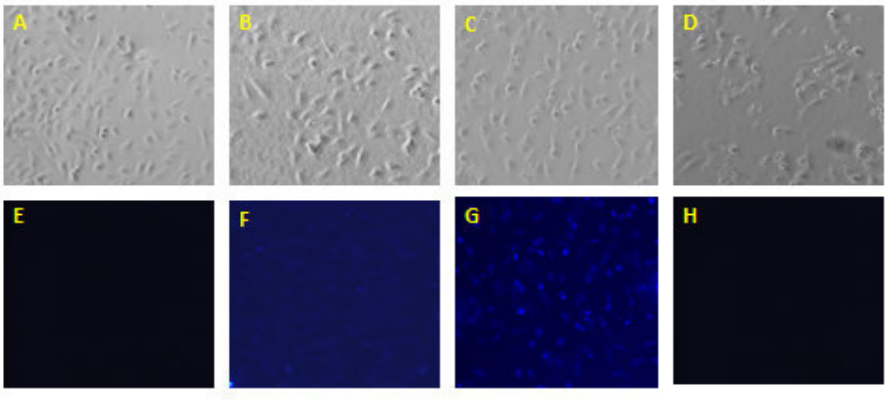

Fig. 13: The images were taken in an inverted fluorescence microscope (Leica DMI6000B) under 20X objective. A) Phase contrast image of the control cells; B) Phase contrast image of the cells treated with **L** (0.011μM) only; C) Phase contrast image of the cells treated with both **L** (0.011μM) and Zn^{2+} (25μM). D) Phase contrast image of the cells treated with Zn^{2+} (25μM) only;E) Fluorescence image of the control cells under UV filter; F) Fluorescence image of the cells treated with **L** (0.011μM) for 2 hours. G) Fluorescence image of the cells treated with **L** (0.011μM) for 2hours and Zn^{2+} (25μM) for 1 hour. H) Fluorescence image of the cells treated with Zn^{2+} only.

In conclusion, we have successfully developed a low cost and easy to synthesize Schiff-base receptor **L** for the selective detection of Zn^{2+} showing 'turn-on' fluorescent response with the detection limit of 16 μM without any interference from other tested metal ions. The receptor **L** can also be used as a chromogenic sensor owing to the noticeable colour change in the presence of Zn^{2+} ions.

3.Experimental

3.1 Materials and methods

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Chemicals were purchased from Sigma Aldrich and were used without further purification.The fluorescence and UV-Visible spectra were recorded on a Fluoromax-4 spectrofluorometer with a 5 nm slit width and a Shimadzu UV-24500 spectrophotometer. Thenitrate salts of the metal ions were used in this study. Ultrapure water with a Millipore Purification System (Milli-Q water) was used throughout the analytical experiments. The ^{1}H-NMR spectra were recorded on a Varian NMR mercury System 300 spectrometer operating at 300 MHz in DMSO-d_6 and IR spectra were recorded on a Perkin Elmer 27 spectrometer in the solid state by preparing KBr discs.

All stock and working solutions were prepared in ultrapure water and with spectroscopic grade MeCN. A stock solution of receptor **L** (1.0 mM) in MeCN was prepared and the corresponding working solution (0.1mM) was prepared simply by diluting with MeCN/H_2O (99:1, *v/v*). Stock solutions of cations (10 mM) in H_2O were prepared and the corresponding working solutions (1 mM) were prepared by diluting with MeCN/H_2O (99:1, *v/v*).

3.2 Synthesis of receptor L

Receptor **L**was synthesized by reacting one mole of 1,2-diaminoethane with (0.6 g, 10 mM) with two moles of 1-(2-hydroxyphenyl)ethanone(2.72 g, 20 mM) in ethanol. The mixture was stirred and refluxed for 3 hrs. Receptor **L** was obtained in good yield and having appearance of yellow bright coloured crystals. 86 %, m.p. 130-131^{0}C. ^{1}H-NMR (400 MHz, $CDCl_3$, ppm) δ=2.40 (s, 6H, 2-CH_3), 4.00 (s, 4H, 2-CH_2), 6.81 (t, 2H, -Ar-H), 6.92-6.94(d2H, Ar-H), 7.28(t, 2H, Ar-H), 7.53-7.55(d, 2H, Ar-H), 15.94(s, 2H, Ar-OH).^{13}C NMR (100 MHz, $CDCl_3$, ppm) δ =12.7, 44.9, 70.5, 76.6, 97.4, 105.2, 108.7, 121.5, 122.0, 124.3, 126.3, 133.8, 135.9, 151.8, 153.6, 164.5, 164.6; LC-MS: [(M+H^+), $C_{18}H_{19}N_3OS$]= 297.10 found 297.20.

3.3Spectroscopic analysis

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The fluorescence titration experiments were carried out with a Fluoromax-4 spectrofluorometer in MeCN/H_2O solvent system at room temperature (298 K) with the aim of determining the binding constant (K_a) for receptor **L.**Zn(II) ion in this solvent system. The fluorescence intensity was recorded at λ_{ex}= 320 nm alongside a reagent blank. The excitation and emission slits were both set to 5.0 nm.These titration experiments were accomplished through a stepwise addition of metal salt solutions (0.02 ml, 1mM, guest) to a solution of receptor **L** (2 ml, 0.1mM, host) in MeCN/H_2O (99:1, *v/v*) in the cell. Simultaneously, the UV-visible titration experiments were carried out in MeCN/H_2O (99:1, *v/v*) solvent system at room temperatures to determine the cations selectivity of **L**in the ground state.

3.4. Computational method

All computations were performed using the Gaussian09W program [27]. Full geometry optimization of **L** and its Zn^{2+} complex were carried out using the DFT method at the B3LYP level of theory in the gas phase. The 6-31G(d,p) basis set was assigned for C, H, N and O atoms. The LANL2DZ basis set with effective core potential was employed for the Zn atom. The vibrational frequency calculations were performed to ensure that the optimized geometries represent the local minima of potential energy surface.

3.5. Cell Culture:

HeLa cells were procured from NCCS, Pune (India) and grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 1% L-glutamine-penicillin-*Streptamycin* at 37 °C and 5% CO_2. Cells were placed on glass coverslips in 12-well plate and allowed to adhere for overnight.

3.6. Cellular Imaging:

At the time of experiment, complete media was replaced with serum free medium. The cells were incubated with **L** (0.011μM) for 2 hours. After 2 hours of incubation with **L**, the cells were then incubated with Zn^{2+} (25μM) for further 1 hour. The cells were washed

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twice with Phosphate Buffer Saline (1X PBS) and then fixed with 100% methanol for 5 minutes and again 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.

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Highlights

- Synthesis of 2-hydroxyacetophenone functionalized Schiff base receptor.
- DFT calculations performed.
- Recognition and sensing of metal ions.
- Fluorescent "turn-on" sensor for Zn^{2+}.
- *In vitro* cell imaging assay.

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Graphical Abstract -Synopsis

A novel linear Schiff-based receptor (**L**) containing two imino phenol units was developed for the selective fluorescent sensing of Zn^{2+} with a discriminating enhancement of 12 folds over the other surveyed cations. Within the huge plethora of Zn^{2+}receptors, this receptor is quite worth to mention because of its simple synthesis, low cost, no interference of Cd^{2+} and the judicious response availed with discernment. The spectroscopic (UV-Vis, fluorescence and mass) results, few mathematical models for determining analytical parameters such as binding constant (K_a), binding stoichiometry, detection limit etc and the frontier molecular orbitals were analyzed to explore the sensing ability of **L** and the mechanism.

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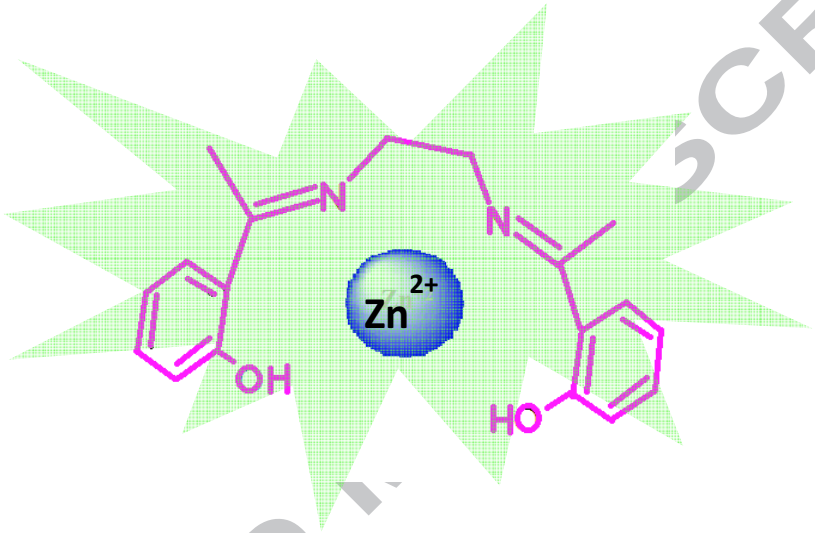
GRAPHICAL ABSTRACT

A novel fluorescent "turn-on" sensor for the biologically active Zn^{2+} ion



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