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An azine based sensor for selective detection of Cu^{2+} ions and its copper complex for sensing of phosphate ions in physiological conditions and in living cells

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ABSTRACT

A simple and cost effective unsymmetrical azine based Schiff base, 5-diethylamino-2-[(2-hydroxy-benzylidene) hydrazonomethyl]-phenol (1) was synthesized which selectively detect Cu^{2+} ions in the presence of other competitive ions through "naked eye" in physiological conditions (EtOH–buffer (1:1, v/v, HEPES 10 mM, pH = 7.4)). The presence of Cu^{2+} induce color change from light yellow green to yellow with the appearance of a new band at 450 nm in UV–Vis spectra of Schiff base 1. The fluorescence of Schiff base 1 (10 μM) was quenched completely in the presence of 2.7 equiv. of Cu^{2+} ions. Sub-micromolar limit of detection (LOD = 3.4×10^{-7} M), efficient Stern–Volmer quenching constant ($K_{SV} = 1.8 \times 10^5$ L mol^{-1}) and strong binding constant (log $K_b = 5.92$) has been determined with the help of fluorescence titration profile. Further, 1 — Cu^{2+} complex was employed for the detection of phosphate ions (PO_4^{3-}, HPO_4^{2-} and $H_2PO_4^-$) at micromolar concentrations in EtOH–buffer of pH 7.4 based on fluorescence recovery due to the binding of Cu^{2+} with phosphate ions. Solubility at low concentration in aqueous medium, longer excitation (406 nm) and emission wavelength (537 nm), and biocompatibility of Schiff base 1 formulates its use in live cell imaging.

1. Introduction

Copper is one of the most essential trace elements of importance for both physical and mental health and serve as a key factor for a wide variety of enzymes in living organisms [1]. However, its excess amount may lead to vomiting, lethargy, increased blood pressure and respiratory rates, acute hemolytic anemia, liver damage, neurotoxicity and neurodegenerative diseases [2,3]. Phosphate ions play essential roles in genetic information storage, gene regulation, energy transduction, signaling processing and muscle contraction [4,5]. Phosphate is a key unit of DNA, RNA and many chemotherapeutic and antiviral drugs [6,7]. In contrast, the over-use of phosphate can lead to excessive algal growth, followed by decomposition and depletion of dissolved oxygen, and finally, the eutrophication of aquatic ecosystems [8]. Hence, much attention has been paid to the development of highly selective and sensitive copper and phosphate sensors for biological and environmental applications.

Although, a number of papers have been published for the detection of copper and phosphate ions, however, there is still an urgent need for simple, biocompatible and cost effective synthetic compounds which may detect copper and phosphate ions with high affinity in competitive aqueous media at physiological conditions [9,10]. Aqueous medium is always interfering in the detection of ions due to their high degree of hydration in water [11,12]. Hence, synthesis of a novel receptor that can detect these ions selectively and sensitively in aqueous solution will be attention-grabbing due to their biological application in live cell imaging.

There are numerous reports in literature enlightening the photochemical behavior and sensing applications of azine based Schiff bases. For example, aldazine based chromo-fluorogenic sensor for fluoride ions in DMSO was developed by Li et al. [13]. Guchhait et al. have investigated spectral properties and sensing ability towards protic environment of a simple azine Schiff base [14]. This sensor is found to be nonfluorescent in aprotic solvents (MeCN and Dioxane) and become highly fluorescent in protic solvents (MeOH and H_2O) due to the formation of intermolecular hydrogen bonding between sensor and protic solvents. However, most of the reported azine based Schiff bases are symmetrical type (RHC═N-N═CHR) and shows aggregation induced emission (AIE) in aqueous solution [15]. A very small amount of research work in literature for unsymmetrical azine based Schiff bases (RHC═N-N═CHR$_1$) motivate us to synthesize these Schiff bases [16]. The designing and synthesis of novel unsymmetrical azine based Schiff base will be interesting because introduction of asymmetry may tune the photochemical properties of synthesized Schiff base.

In this work unsymmetrical azine based Schiff base, 5-diethylamino-2-[(2-hydroxy-benzylidene)hydrazonomethyl]-phenol (1) was synthesized in excellent yield from inexpensive and biocompatible starting

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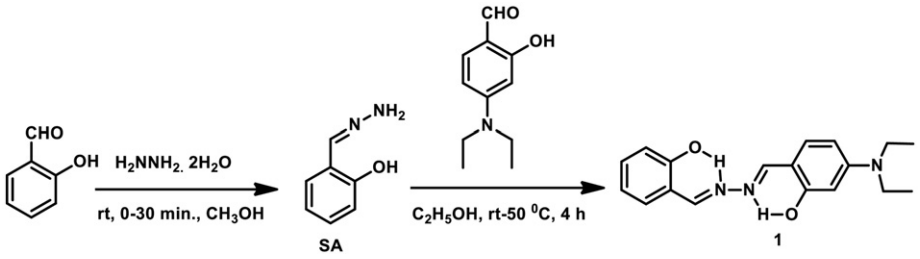


Scheme 1. Synthesis of Schiff base 1.

materials having 2-hydroxysalicylaldehyde as main component. The Schiff base 1 possess both charge transfer and proton transfer group and was found to be highly fluorescent in both organic and aqueous solution. Coupled excited state intramolecular proton transfer (ESIPT) and intramolecular charge transfer (ICT) have been widely explored in the molecular systems possessing both charge transfer and proton transfer groups. However, there are a very small number of reports in literature, where one process is suppressed by other viz. Guchhait et al. have observed the ICT suppressed ESIPT single fluorescence at 527 nm for 4-(diethylamino)-2-hydroxybenzaldehyde [17]. In the case of Schiff base 1, ICT is suppressed and only ESIPT is observed because uneven intramolecular charge transfer facilitates ESIPT.

Most of the reported azine based Schiff base chromo- fluorogenic sensors for Cu^{2+} suffer from the interferences caused by Fe^{3+}, poor water solubility, use of organic solvents and poor detection limit [18, 19]. However, this chromo-fluorogenic Schiff base (1), synthesized by simple condensation reactions, is very efficient in selective detection of Cu^{2+} in EtOH–buffer solution (1:1, v/v, HEPES 10 mM, pH = 7.4) with LOD = 3.4×10^{-7} M. Further, 1 — Cu^{2+} complex was also utilized for sensing of phosphate ions in the same aqueous solution. Logic gate were constructed successfully by taking two input Cu^{2+} and EDTA in aqueous solution and a number of reversible cycles were observed upon alternative addition of Cu^{2+} and EDTA in EtOH–buffer (1:1, v/v, HEPES 10 mM, pH = 7.4) solution of Schiff base 1. To further evaluate the biological application of Schiff base 1, fluorescence has also been employed for detection of these ions in living cells.

2. Experimental

2.1. Materials and Methods

All analytical reagent grade chemicals were obtained from the commercial sources. Perchlorate salts of cations, tertabutylammonium salts of anions and HEPES buffer were purchased from Sigma-Aldrich Chemicals, USA. Di sodium hydrogen phosphate dehydrate, Potassium dihydrogen orthophosphate, Aluminium(III) nitrate, tetrasodium EDTA (ethylene diamine tetra acetic acid) and all the solvents were obtained from Merck Chemicals, India. Absolute ethanol (Emsure grade) was purchased from Merck Germany. All the solvents were used after checking their purity thoroughly by UV–Vis and fluorescence spectral techniques. Double distilled water was prepared in lab using double distillation unit.

2.2. Synthesis of Schiff Base 1

The Schiff base, 5-diethylamino-2-[(2-hydroxy-benzylidene) hydrazonomethyl]-phenol (1) was synthesized by two step condensation reactions (Scheme 1). Synthetic methodology involved initial stirring of hydrazine hydrate with 2-hydroxybenzaldehyde in dry methanol at room temperature for 30 min to yield 2-hydrazonomethyl phenol (SA). The heating of SA with 4-diethylamino-2-hdroxybenzaldehyde in ethanol at 50 °C afforded the corresponding 5-diethylamino-2-[(2-hydroxy-benzylidene)hydrazonomethyl]-phenol (1) in excellent yield.

2.2.1. Analytical Data

$C_{18}H_{21}N_3O_2$: Physical state: Pale yellow solid, Yield 88%; M.p. 160 °C; found % for C, 69.51; H, 6.73; N, 13.55, Calc: C, 69.43; H, 6.80, N, 13.49; ^1H NMR (300 MHz, $CDCl_3$) δ 1.20 (t, J = 6.9 Hz, —CH_3); 3.40 (q, J = 7.2 Hz, 4H, –CH_2); 6.21 (d, J = 2.4 Hz, 1H, Ar—H); 6.27 (dd, J = 8.7, 2.7 Hz, 1H, Ar—H); 6.93 (dt, J = 7.8, 1.2 Hz, 1H, Ar—H); 7.00 (d, J = 8.1 Hz, 1H, Ar—H); 7.12 (d, J = 8.7 Hz, 1H, Ar—H); 7.29–7.35 (m, 2H, Ar—H); 8.52 (s, 1H, Iminic-H); 8.60 (s, 1H, Iminic-H), 11.64 (s, 1H, —OH, exchangeable with D_2O), 11.66 (s, 1H, –OH, exchangeable with D_2O) ^{13}C NMR (75 MHz, $CDCl_3$) δ 12.55, 44.47, 97.80, 104.30, 106.38, 116.91, 117.99, 119.59, 131.91, 132.51, 133.95, 152.00, 159.50, 161.16, 161.97, 163.91; HRMS Calc. $[M + H]^+$ 312.16 found 311.38 (100% abundance).

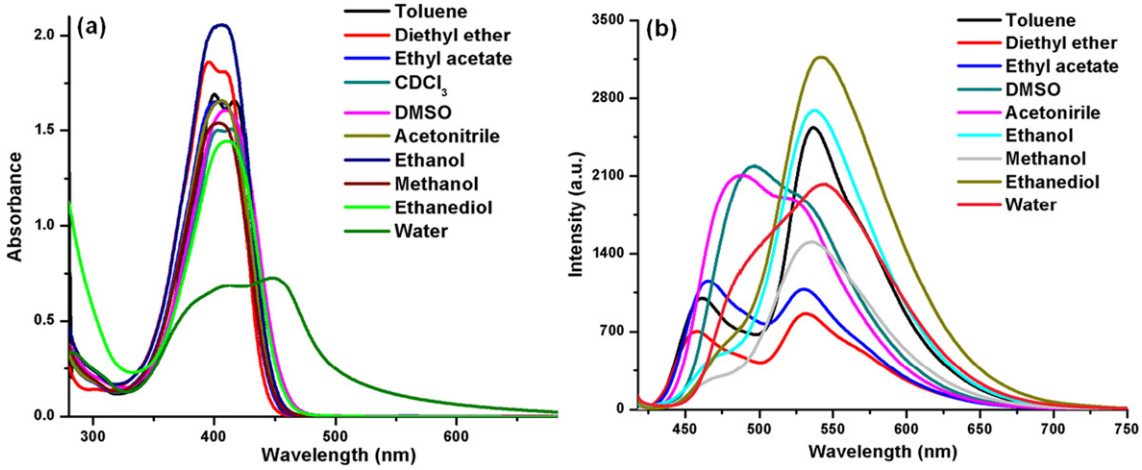


Fig. 1. Changes in UV–Vis spectra (a) and fluorescence spectra (b) of Schiff base 1 (30 μM) in different solvents with varying polarities and hydrogen bonding abilities.

Table 1
UV–Vis absorption and fluorescence spectral maxima of Schiff base 1 in various solvents, and solvent polarity parameters. Where, α is solvent hydrogen-bond donor acidity, β is solvent hydrogen-bond acceptor basicity and π* is a measure of the solvent dipolarity/polarizability.

Solvents	Schiff base 1		$E_T(30)$[a]	α[b]	β[b]	π*[b]
	λ_{abs} (nm)	λ_{em} nm (intensity) λ_{ex} = 406 nm				
Toluene	400, 416	461 (999), 536 (2530)	33.9	0.0	0.13	0.54
Diethyl ether	394, 410	457 (700), 531 (862)	34.6	0.0	0.47	0.27
Ethyl acetate	403	465 (1156), 530 (1079)	38.1	0.0	0.45	0.55
DMSO	409	496 (2191), 530 (1901)	45.1	0.0	0.76	1.00
Acetonitrile	406	488 (2106), 526 (1866)	45.9	0.19	0.40	0.75
Ethanol	406	464 (414), 536 (2688)	51.9	0.86	0.75	0.54
Methanol	404	467 (269), 535 (1505)	55.5	0.98	0.66	0.60
Ethanediol	412	472 (513), 542 (3166)	56.3	0.90	0.52	0.92
Water	414, 449	484 (1167), 542 (2023)	63.1	1.17	0.47	1.09

[a] Values obtained from the literature [27], given in kilocalories per mole.
[b] Values obtained from the literature [28,29].

2.3. Instrumentation

^1H and ^{13}C NMR spectra were recorded in $CDCl_3$ on a JEOL AL-300 FT-NMR multinuclear spectrometer. Chemical shifts were reported in parts per million (ppm) using tetramethylsilane (TMS) as an internal standard. UV–Vis spectra were recorded by Shimadzu spectrophotometer, model, Pharmaspec UV–1800. Fluorescence spectra were recorded on a Hitachi, F-4600 fluorescence spectrophotometer. Time resolved fluorescence was performed with a HORIBA time resolved fluorescence spectrophotometer. Mass spectrometric analysis was carried out on a Water-Q-Tof micromass equipment using ESI as the source.

2.4. General Methods

UV –Vis and fluorescence experiments were performed at room temperature. For UV –Vis and fluorescence titration 10 μM solution of Schiff base 1 in ethanol/ethanol-buffer solution (1:1, v/v, HEPES 10 mM, pH = 7.4) was used. 10 mM HEPES buffer was prepared in double distilled water. The solutions of metal perchlorate and tetrabutylammonimum anion were prepared in ethanol –water (1:1, v/v). Donor-receptor binding ratio was determined by Job's plots, while binding constant (log K) of the donor-receptor adduct was analyzed by linear fitting of UV –Vis/fluorescence titration curve in the following equation (Eq. (1)) [20].

$$\log[\ A - A_{min}/A_{max} - A] = \log K + \log\left[G^{n\pm}\right] \quad (1)$$

where, A and A_{min} are absorbance/fluorescence of Schiff base 1 at particular wavelength, in the presence and absence of guest ions and A_{max} is the maximum absorbance/fluorescence in the presence of guest ions. The Stern–Volmer Eq. (2) was applied for the calculation of quenching constant [21].

$$I_o/I = 1 + K_{SV}\left[Cu^{2+}\right] \quad (2)$$

where, I_o and I are the fluorescence intensity in the absence and presence of Cu^{2+} ions. K_{SV} is the Stern–Volmer quenching constant and $[Cu^{2+}]$ is the concentration of copper ions. Cu^{2+} binds to Schiff base 1 and forms 1 — Cu^{2+} complex, accordingly, the binding number of Cu^{2+} in Schiff base 1 was calculated by using double–logarithmic Eq. (3) [21].

$$\log(I_o - I/I) = \log K_b + n\ \log\left[Cu^{2+}\right] \quad (3)$$

where, n is the binding number, and log K_b is the binding constant.

The limit of detection (LOD) was determined from a plot of absorbance/fluorescence intensity as a function of the concentration of the added ions. To determine the S/N ratio, the absorbance/fluorescence intensity of receptor without ions was measured by 10 times and the standard deviation of blank measurements was determined. The detection was calculated as three times the standard deviation from the blank measurement (in the absence of ions) divided by the slope of calibration plot between ion concentration and absorbance/fluorescence intensity [22].

Quantum yield was calculated by using following equation:

$$Q = Q_R\ (I/I_R) \times (OD_R/OD) \times \left(n^2/n_R{}^2\right) \quad (4)$$

where, Q is fluorescence quantum yield, I is the integrated fluorescence intensity, n is the refractive index of solvent, and OD is the optical density (absorption). The subscript R refers to the reference quinine sulfate of known quantum yield (0.54 in 0.1 M H_2SO_4). The excitation wavelength was set at 358 nm which was the UV–Vis crossing point obtained by plotting UV–Vis of Schiff base 1 in absolute ethanol and quinine sulfate in 0.1 M H_2SO_4 (OD was kept at 0.1 at 358 nm).

2.5. Details of Biological Study

2.5.1. Materials

Dulbecco Modified Eagle Medium (DMEM), trypsin and DMSO were purchased from Himedia, India. Fetal Bovine Serum (FBS) from Biological Industries Ltd. Israel. Antibiotic solution (Penicillin 1000 IU and

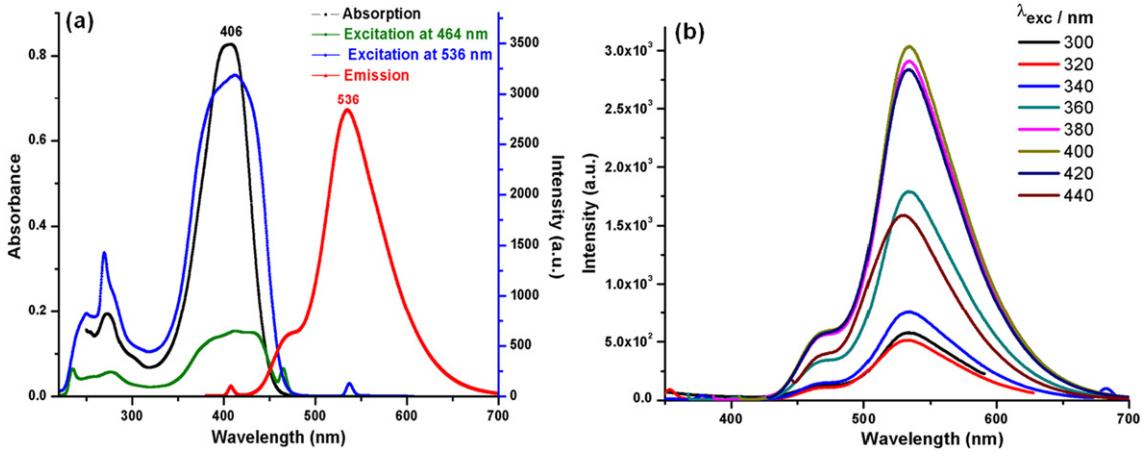


Fig. 2. (a) UV–Vis absorption, fluorescence excitation and fluorescnce emission spectra, and (b) Fluorescence spectra at different excitation wavelengths of Schiff base 1 in ethanol.

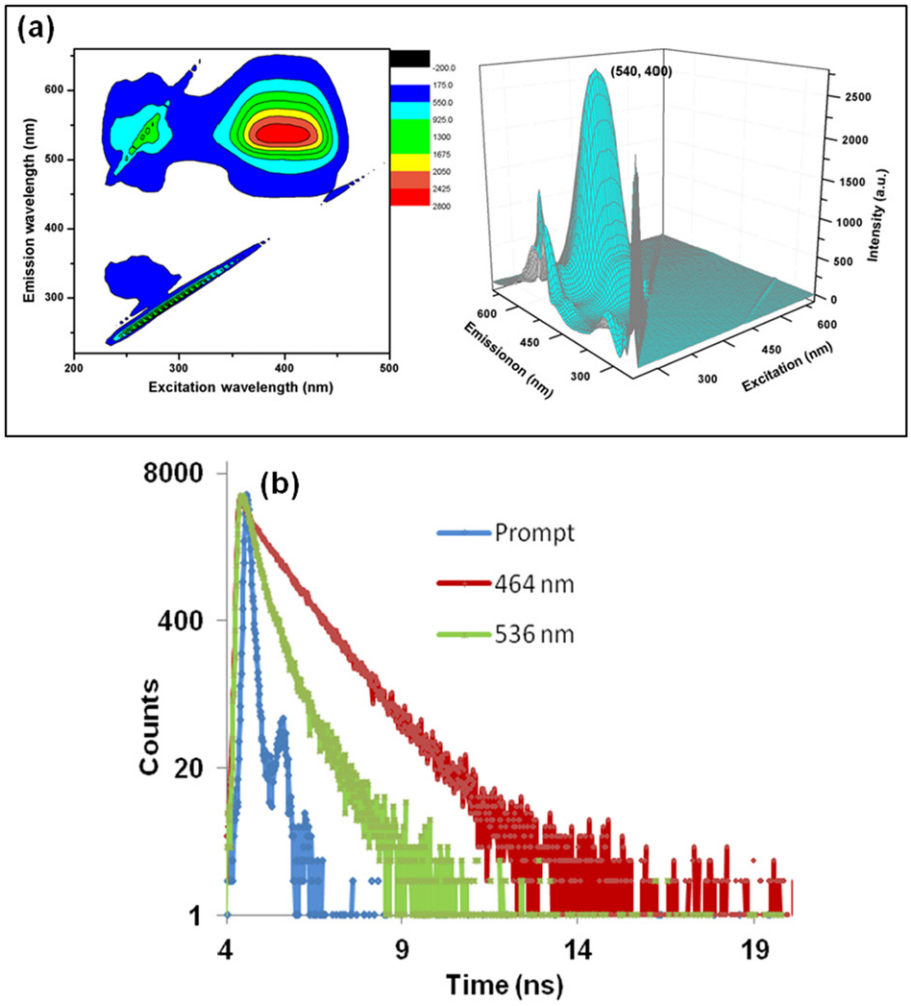


Fig. 3. 3D spectra contour plot and corresponding three dimensional projections and (b) Time resolved fluorescence spectra of Schiff base 1 in ethanol ($\lambda_{exc} = 377$ nm and $\lambda_{em} = 464$ nm & 536 nm).

Streptomycin 10 mg/mL and Gentamycin), MTT(3–(4,5–dimethylthiazol–2–yl)–2,5-diphenyltetrazoliumbromidedye) were obtained Sigma-Aldrich Chemicals, USA.

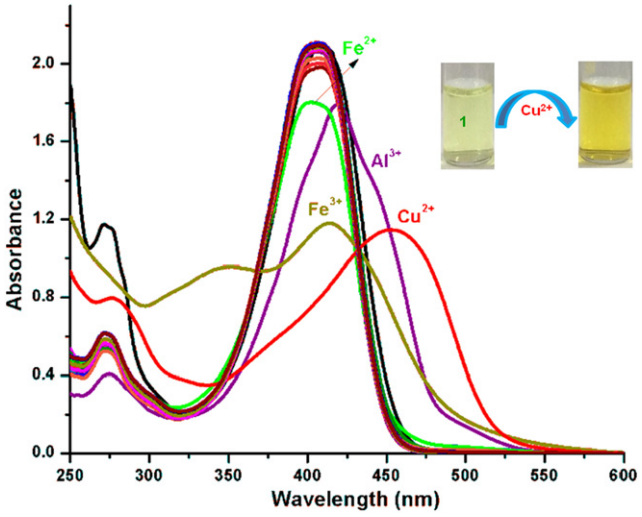


Fig. 4. Changes in UV–Vis spectra of Schiff base 1 (30 μM) upon addition of 300 μM of various ions (Li^+, Na^+, Al^{3+}, Pb^{2+}, Fe^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Hg^{2+}, Br^-,Cl^-, HSO_4^-, F^-, SCN^-, CH_3COO^-, CN^-, $H_2PO_4^-$, HPO_4^{2-} and PO_4^{3-}) in ethanol.

2.5.2. Cell Culture

A549 cancerous cells were cultured in DMEM solution which was prepared by mixing of 2.70 g DMEM, 0.3 g $NaHCO_3$, 0.24 g Penicillin, 0.1 g Streptomycin, 500 μL Gentamycin, 20 mL FBS, 180 mL autoclaved water. The cells were incubated in a humidified atmosphere at 37 °C in 5% CO_2. Stock solution of Schiff base 1 was prepared in DMSO which was further diluted with DMEM.

2.5.3. MTT Assay

For cytotoxic analysis A549 cancerous cells were seeded at the density of 5×10^3 cells per well. Further, cells were incubated in the presence of different concentrations of Schiff base 1 (5 μM, 10 μM, 20 μM and 50 μM) for 24 h at 37 °C in 5% CO_2. After treatment, 100 μL of 0.5 mg/mL MTT in incomplete growth medium was added and the plate was re-incubated for 2–4 h. Supernatant was discarded and water insoluble formazan crystals were dissolved in 100 μL of DMSO by mixing for 10 min. The optical density (O.D.) was taken at 595 nm at Biotech Synergy HT MultiMode Microplate reader.

2.5.4. Cell Imaging

A549 cells (10^5 cells/mL/well) were seeded in a six well tissue culture plate over coverslip in each well and allowed to adhere overnight. Thereafter, the cells were incubated with 5 μM Schiff base 1 for 2 h. For detection of Cu^{2+} and PO_4^{3-}, pre-treated cells were treated for 30 min with Cu^{2+}. The cells, pre-treated with Schiff base 1 + Cu^{2+} were then

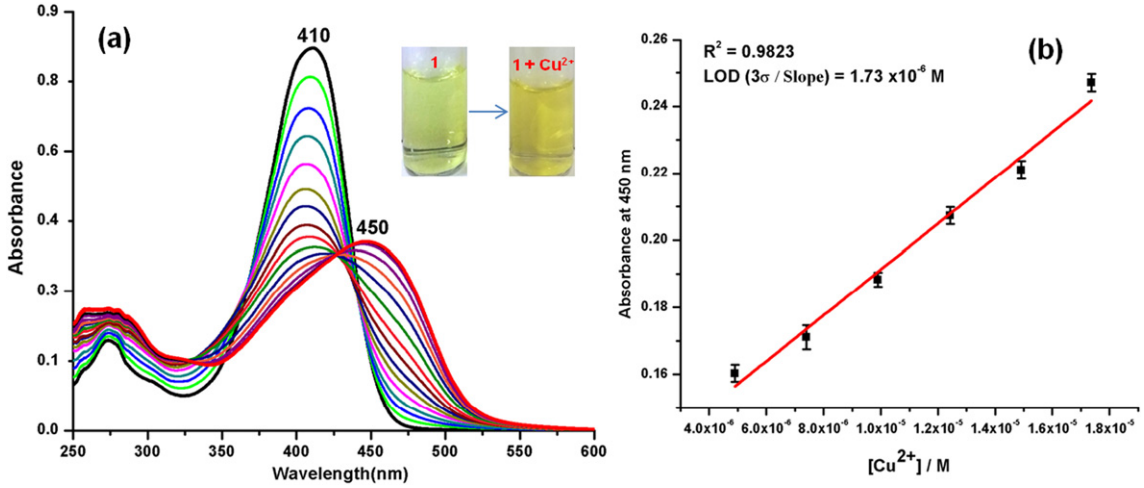


Fig. 5. (a) Changes in UV–Vis spectra of Schiff base 1 (10 μM) upon gradual addition of Cu^{2+} (0–39.7 μM) in ethanol–buffer solution (1: 1, v/v, 10 mM HEPES, pH = 7.4) (b) Limit of detection (LOD) curve plot. The error bars represent the standard deviation of three independent measurements.

treated subsequently with Cu^{2+} and PO_4^{3-} for 30 min each. For cell imaging PO_4^{3-} was taken as representative ions among other phosphate ions. After treatment, cells were washed thoroughly with chilled phosphate buffer saline (PBS) and fixed with chilled 4% paraformaldehyde for 20 min and images were recorded on Nikon Air Laser Scanning Confocal Microscope System for analyzing the fluorescence changes.

3. Results and Discussion

3.1. Synthesis and Structural Characterization of Schiff Base 1

The receptor, 5-diethylamino-2-[(2-hydroxy-benzylidene) hydrazonomethyl]-phenol (1) was obtained with a high yield by the reaction between 2-hydrazonomethyl phenol and 2–4-diethylamino-2-hdroxybenzaldehyde in ethanol in 1:1 molar ratio. Schiff base 1 is highly stable pale-yellow solid and was well characterized by using physicochemical and spectroscopic tools viz. NMR and mass spectra. Since Schiff base 1 contain hydroxyl groups it may show unusual properties in solution phase due to the existence of O—H· · ·N or O· · ·H—N type hydrogen bonds and tautomerism between enol and keto forms (Fig. S1). Therefore, it is important to investigate its possible form in solution state. The ^1H NMR spectrum showed two sharp singlet, at δ = 11.66 ppm and 11.64 ppm these are assigned to two −OH proton, indicating existence of Schiff base 1 in enol form in $CDCl_3$ solution (Fig. S2). Two singlet observed for two >CH═N— at 8.52 ppm and 8.60 ppm, clearly proves the formation of Schiff base 1. In ^{13}C NMR spectrum two imine carbons also showed an expected chemical shift value of δ = 159.50 ppm and 161.16 ppm (Fig. S3). Peak at *m/z* of 312.1705 in HRMS corresponding to $[M + H]^+$ also validate the molecular structure of Schiff base 1 (Fig. S4).

3.2. UV–Vis and Fluorescence Studies

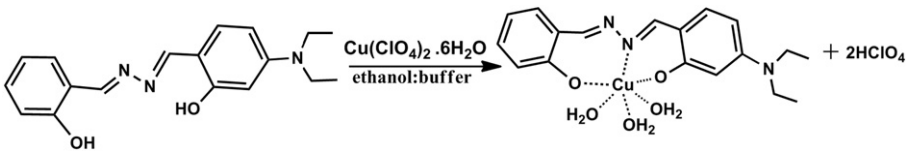
3.2.1. Effects of Solvents on UV–Vis and Fluorescence Spectra of Schiff Base 1

The UV–Vis and or fluorescence spectra of Schiff base 1 (30 μM) were examined at room temperature in a series of polar, nonpolar, protic and aprotic solvents. Schiff base 1 exhibited yellowish green color, visible to naked eye and displayed an absorption band in the range 400–449 nm in all the solvents (Fig. 1, Table 1). The high absorbance for the band in the region 400–449 nm indicates it may be π → π* type transition. The UV–Vis spectra showed less pronounced changes in wavelength position (394–416 nm) upon varying the polarity or protocity of solvents except in case of water showing a red shifted split band at 414–449 nm.

The fluorescence spectra were recorded in different solvents by exciting the Schiff base 1 at its λ_{max} absorption 406 nm and the results are displayed in Fig. 1. The fluorescence spectra showed two well resolved bands in all the solvents, shorter wavelength fluorescence (B band) in the range 464–494 nm and longer wavelength fluorescence (A band) in the range 520–542 nm (Table 1). The intensity ratio of I_A/I_B is found to vary significantly in different solvents. In aprotic solvents (toluene) and polar protic solvents (ethanol, methanol, water and ethanediol) band A is found to be more intense than band B. However, in DMSO, acetonirile and ethyl acetate, band B becomes more intense. Diethyl ether showed equal abundance of both the bands in solution. The variation in intensity, I_A/I_B may be due to the differential stabilization of both the emission bands by different solvents at excited state. The shorter wavelength fluorescence was found to red shifted in DMSO, acetonirile, water and ethanediol at 496, 488, 484 and 472 nm respectively. Although, longer wavelength fluorescence (530–536) showed less pronounced changes at band position in most of the tested solvents, however, in water and ethanediol red shifted band at 542 nm and in acetonitrile blue shifted band at 526 nm was observed.

3.2.2. Nature of UV–Vis Bands

Since in Schiff base 1 proton transfer groups are present, therefore this molecule may show keto–enol tautomerization at ground state. Ethanol has been assigned as representative solvent for recording the spectra of various reference compounds to go insight the UV–Vis spectra of Schiff base 1.The UV–Vis spectra of salicylaldehyde exhibited two bands at 324 nm and 255 nm (Fig. S5). The low energy band at 324 nm may originate from the transition $S_0 \rightarrow S_1$ ($\pi \rightarrow \pi^*$) of the intramolecular hydrogen bonded salicylaldehyde and high energy band may assigned to the $S_0 \rightarrow S_2$ transition. Monohydrazone of salicylaldehyde,



Scheme 2. Schematic diagram representing binding interactions of Schiff base 1 with Cu^{2+}.

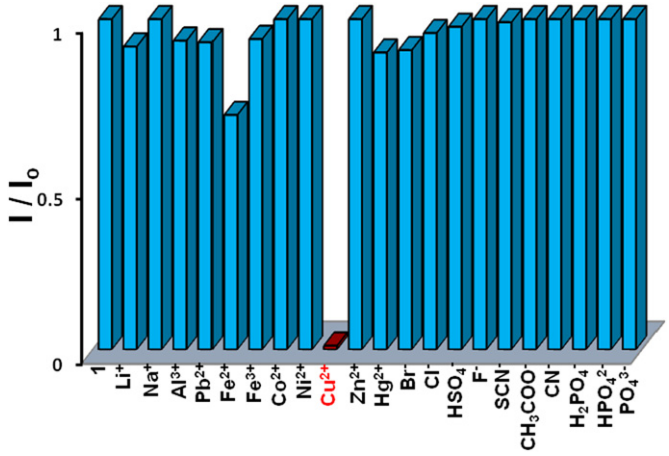


Fig. 6. Changes in fluorescence spectra of 30 μM Schiff base 1 upon addition of 2 equiv. of various ions (from left, 1, Li^+, Na^+, Al^{3+}, Pb^{2+}, Fe^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Hg^{2+}, Br^-,Cl, HSO_4^-, F^-, SCN^-, CH_3COO^-, CN^-, $H_2PO_4^-$, HPO_4^{2-} and PO_4^{3-}) in ethanol-buffer solution (1:1, v/v, 10 mM HEPES, pH = 7.4), λ_{exc} = 406 nm and λ_{em} = 537 nm. Where, I_o & I is the fluorescence intensity at λ_{max} = 537 in the absence and presence of ions, respectively.

SA (318 & 272 nm) and dihydrazone (357 & 293 nm) of salicylaldehyde (SAA) showed bands for enol form only and no keto form (generally observed at higher wavelength > 400 nm) is found to be present. Ziolek et al. also observed the similar results for SAA and explained that the keto–enol equilibrium cannot be formed at ground state due to the strong intramolecular hydrogen bonding in SAA [23]. Unsymmetrical introduction of charge transfer moiety in SAA (Schiff base 1) showed drastically red shifted broad absorption band at 406 nm as mentioned earlier. Now to know whether this band in Schiff base 1 is keto/enol type UV–Vis spectra in various solvents were examined carefully. The insignificant polarity and protocity dependency of this band insist us to assign it as enolic band. Moreover, ^{1}H NMR of Schiff base 1 showed the presence of only enol form in $CDCl_3$ solution. Therefore, we have also recorded UV–Vis spectra of Schiff base 1 in $CDCl_3$ and found a very similar split band at 402 and 414 nm (Fig. 1). These findings clearly proofs that this is the enol form which is found to exist in different solvents. The absence of keto tautomer in solvents shows that the enol tautomer is energetically much stabilized than keto tautomer and hence, keto-enol tautomerization could not observed in the ground state.

Now if this band is due to intramolecular charge transfer from diethyl amino group to imine group then in protic solvents it must show blue shift due to the intermolecular hydrogen bonding between amine nitrogen and water molecule. However, in water it is showing a red shifted band at 414–449 nm this may be explained as: there are three hydrogen bond acceptor (HBA) site in Schiff base 1 viz. nitrogen atom of —NEt_2 and —C═N group, and oxygen atoms of hydroxyl groups. Charge density at oxygen atoms is higher compared to —NEt_2 owing to the presence of nearby hydrogen bond acceptor imine group. In the ground state —NEt_2 group is expected to be co-planar with aromatic ring and therefore it can delocalize its charge density with the π cloud of the aromatic ring. This may decrease the charge density at —NEt_2 group, however, increases the same at oxygen atoms of hydroxyl groups through resonance effect. Therefore, the water, strongest HBD (hydrogen bond donor) type solvent prefers oxygen atoms for strong intermolecular hydrogen bonding [24]. Consequently, the Schiff base 1 is energetically more stabilized and shows red shifted UV–Vis spectra in water.

3.2.3. Nature of Fluorescence Bands

Both charge transfer and proton transfer moieties are present in Schiff base 1, therefore, LE (local emission), ICT (intramolecular charge transfer) and PT (proton transfer) can be observed in this molecule. The observation of two different fluorescence bands for a molecule is either due to two different ground state species or one single species that undergoes a excited state reaction (for example, proton transfer and twisted intramolecular charge transfer (TICT) etc.). The fluorescence spectra of Schiff base 1 in representative solvent, ethanol exhibited two bands at 536 and 464 nm. The fluorescence excitation spectra monitored either at 536 or 464 nm shows similar band at 412 nm except for the difference in intensity and well agree with its absorption band at 406 nm Fig. 2. These results provide evidences for the origin of both fluorescence bands through excitation of the same ground state. To confirm this result 3D fluorescence spectral studies were performed and only one contour was observed in ethanol. The corresponding excitation shows a band at 400 nm, and the fluorescence displayed two bands at 538 and 464 nm (Fig. 3). The excitation and 3D fluorescence demonstrate the presence of only one species in ground state. Moreover, excitation independent fluorescence also validates the same (Fig. 2). Since dual fluorescence is not because of two different species in ground state, there may be possibility of excited state reaction upon excitation of Schiff base 1. Time-resolved fluorescence measurement has been performed to investigate the excited state reaction. The decay curve at 464 nm and 536 nm could not well fitted by mono, bi and multi exponential decay pattern with acceptable χ^2 values ($\chi^2 < 1.2$) (Fig. 3) indicating existence of more than one species at excited state. The excited

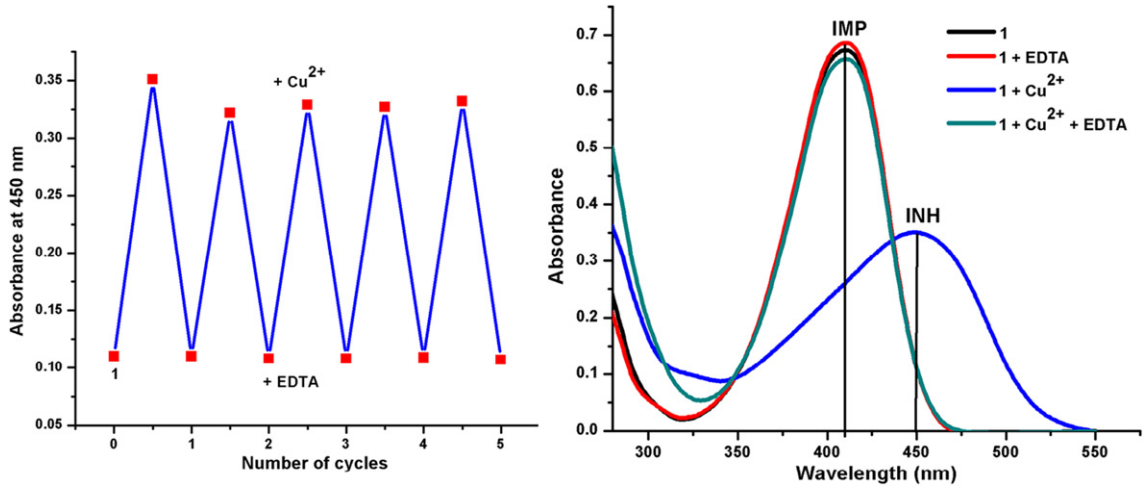


Fig. 7. Reversibility of UV–Vis response of Schiff base 1in ethanol–buffer solution (1:1, v/v, 10 mM HEPES, pH = 7.4) with alternate addition of Cu^{2+} and EDTA (left). Diagram representing molecular logic functions, IMP (410 nm) and INH (450 nm) for Schiff base 1 in the presence of Cu^{2+} and or EDTA (right).

Table 2
Truth table for 10 μM Schiff base1 in ethanol–buffer solution (1:1, v/v, 10 mM HEPES, pH = 7.4) with 10 equiv. Cu^{2+} and 10 equiv. EDTA.

Input		Output	
Cu^{2+}	EDTA	IMP Absorbance at 410 nm	INH Absorbance at 450 nm
0	0	1	0
1	0	0	1
0	1	1	0
1	1	1	0

state reaction that is going on upon excitation may be ESIPT this is because the band at 536 nm was found to be polarity and protocity independent. Charge transfer bands are generally found to be polarity and protocity dependent. Hence, the fluorescence at 464 and 536 nm of Schiff base 1 in ethanol may be assigned as LE and ESIPT fluorescence, respectively.

Although, Schiff base 1 posses both proton transfer and charge transfer groups, however, only ESIPT is observed this may be due to the suppression of ICT by ESIPT. There are various reports in literature explaining coupled ESIPT and ICT reaction in those molecular systems which have both charge transfer and proton transfer groups [25]. However, there are very few reports for these molecular systems, where fluorescence is observed either by ICT or ESIPT. Up to my knowledge till date there is only one report explaining ICT suppressed ESIPT single fluorescence at 527 nm for 4-(diethylamino)-2-hydroxybenzaldehyde [17]. Quantum yield for Schiff base 1 was calculated in ethanol (0.033), acetonitrile (0.017) and in toluene (0.030) by taking quinine sulfate as reference.

3.2.4. Effects of Ions on UV–Vis and Fluorescence Spectra of Schiff Base 1

Insignificant changes are observed in UV–Vis spectra of Schiff base 1 upon varying the polarity and protocity of solvents. However, interactions with different ions may bring some interesting findings. Therefore, the molecular interactions of Schiff base 1 with Li^{+}, Na^{+}, Al^{3+}, Pb^{2+}, Fe^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Hg^{2+}, Br^{-}, Cl^{-}, HSO_4^{-}, F^{-}, SCN^{-}, CH_3COO^{-}, CN^{-}, $H_2PO_4^{-}$, HPO_4^{2-} and PO_4^{3-} were studied initially in ethanol and the results are depicted in Fig. 4. Interestingly, it was found that upon addition of various ions (300 μM) in 30 μM Schiff base 1, drastic changes are observed only in the presence of Cu^{2+}, Al^{3+} and Fe^{3+}. In the presence of Al^{3+} and Fe^{3+} the band at 406 nm is found to be red shifted at 420 nm and 414 nm respectively. A new band at 445 nm is also found to develop in the presence of Al^{3+}. Addition of 300 μM of Cu^{2+} results in appearance of a new band at 455 nm with color change from light yellow green to dark yellow visible to naked eye. Further, solvent optimization studies helps in finding the suitable ethanol–buffer solution (1:1, v/v, HEPES 10 mM, pH = 7.4) for selective and efficient detection of Cu^{2+} in polar protic solvents. Similar to ethanolic solution, upon addition of Cu^{2+} in ethanol–buffer solution (1:1, v/v, 10 mM, pH = 7.4), a new band is observed at 450 nm with the disappearance of 410 nm absorption band (Fig. 5). The appearance of new band may be due the deprotonation and binding of Cu^{2+} with Schiff base 1 (Scheme 2) [26]. Interestingly, presence of Al^{3+} and Fe^{3+} do not show significant changes in UV–Vis spectra of 30 μM ethanol–buffer solution (1:1, v/v, 10 mM, pH = 7.4) of Schiff base 1 which may be due to the high degree of solvation of these ions in aqueous solution (Fig. S6).

The fluorescence changes of Schiff base 1 with aforementioned ions were investigated in ethanol–buffer solution (1:1, v/v, HEPES 10 mM, pH = 7.4). The Schiff base 1 (30 μM) displayed a very intense fluorescence band at 537 nm and a weak intense band at 464 nm when excited at 406 nm. Upon addition of 2 equiv. of various ions, incredible fluorescence quenching at 537 and 464 nm was observed only in the presence of Cu^{2+} ions. Other tested ions do not show significant changes in the fluorescence intensity (Fig. 6).

Hydrolysis of Schiff bases in the presence of even small amount of water in protic solvents is the major concern of issue. Therefore, the temporal absorption changes for the Schiff base 1 in the ethanol–water (1:1, v/v) were measured and found that hydrolysis does not occur (Fig. S7). These results strengthen the stability of Schiff base 1 in ethanol–water (1:1, v/v) for performing spectral studies in this solution mixture.

3.2.5. Colorimetric Sensing of Cu^{2+} Ions

To study the binding interactions of Schiff base 1 (10 μM) with Cu^{2+} ions, UV–Vis titration was performed in ethanol (Fig. S8). With the addition of incremental amount of Cu^{2+} ions (0–44.4 μM), the absorption band at 406 nm diminished gradually with the appearance of newly growing band centered at 455 nm, corresponding to a colorimetric change from light yellow green to yellow. A clear isosbestic point at 430 nm indicates that only two species are in equilibrium. Job's plot indicates the formation of 1:1, 1 — Cu^{2+} complex (Fig. S9). With the help of titration profile association constant (log K) and limit of detection (LOD = 3σ / slope) has been calculated to be 6.710 and 8.5×10^{-7} M, respectively (Figs. S8 & S10). To study the interference of other potentially competitive ions on 1 — Cu^{2+} complexation, UV–Vis competition experiments was performed in the presence of 10 equiv. of Cu^{2+} mixed with 10 equiv. of other ions. Unfortunately, a few ions are found to interfere in the detection of Cu^{2+} in ethanol (Fig. S11).

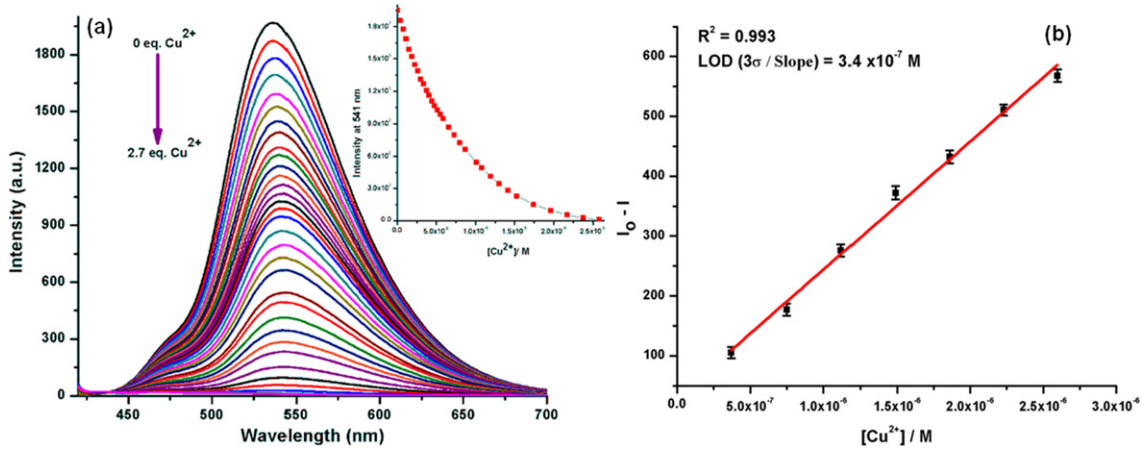


Fig. 8. (a) Fluorescence titration spectra of Schiff base 1 (10 μM) upon incremental addition of 2.7 equiv. of Cu^{2+} in ethanol-buffer solution (1:1, v/v, 10 mM HEPES, pH = 7.4), λ_{exc} = 406 nm and λ_{em} = 537 nm. Inset: Changes in the fluorescence intensity at 541 nm with incremental addition of Cu^{2+}(b) Detection limit curve plot. The error bars represent the standard deviation of three independent measurements.

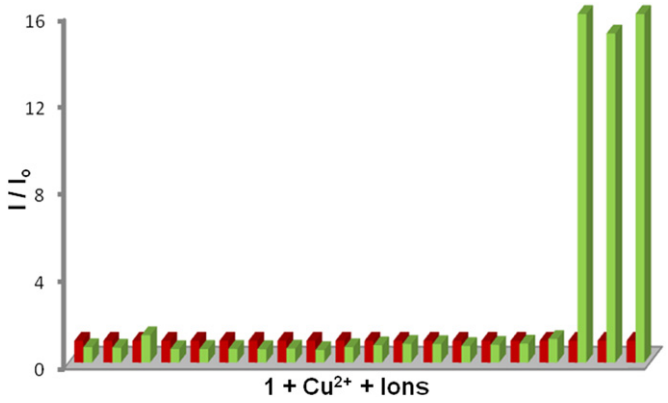


Fig. 9. Bar diagram representing selectivity of Schiff base 1 for Cu^{2+} in ethanol–buffer solution (1:1, v/v, 10 mM HEPES, pH = 7.4). The maroon bars represent the fluorescence of solution of 10 μM Schiff base1 and 10 equiv. of Cu^{2+}. The green bars show the fluorescence change upon addition of 10 equiv. of the corresponding ions (from left, Li^+, Na^+, Al^{3+}, Pb^{2+}, Fe^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Hg^{2+}, Br^-, Cl^-, HSO_4^-, F^-, SCN^-, CH_3COO^-, CN^-, $H_2PO_4^-$, HPO_4^{2-} and PO_4^{3-}) to the mixture solution of 10 μM Schiff base 1 + 10 equiv. of Cu^{2+}. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Further, colorimetric LOD, 1.7×10^{-6} M for Cu^{2+} ions was calculated in 10 μM ethanol–buffer solution (1:1, v/v, HEPES 10 mM, pH = 7.4) of Schiff base 1 with the help of UV–Vis titration profile (Fig. 5). Upon varying the concentration of Cu^{2+} ions (0–39.7 μM), a new band was appeared at 450 nm with an isosbestic point at 428 nm. The competition experiment for the selectivity of Cu^{2+} ions showed insignificant interference by any other ions unusual to ethanolic solution (Fig. S12). The reason may be due to the higher binding affinity of Schiff base 1 towards Cu^{2+} ions than that of other ions in aqueous solution. The calculated LOD in both the solution is much lower than that (3.0×10^{-5} M) recommended by WHO.

The reversibility of Schiff base 1 towards Cu^{2+} was examined by the addition of 100 μM EDTA in a mixture solution of 10 μM Schiff base 1 and 100 μM Cu^{2+}. Addition of EDTA brings back the absorption spectrum (λ_{max} = 410 nm) as well as yellow green color of Schiff base 1. The presence of EDTA recovers the absorption of Schiff base 1 because it has high binding affinity towards Cu^{2+} and forms highly stable EDTA–Cu^{2+} complex. The reversibility remains static with respect to color change and absorbance even after several cycles with the sequentially alternative addition of Cu^{2+} and EDTA (Fig. 7). These results indicate that Schiff base 1 can be employed as a reversible colorimetric sensor for Cu^{2+} ions in ethanol–buffer solution (1:1, v/v, HEPES 10 mM, pH = 7.4).

The change in absorbance at 410 and 450 nm upon addition of Cu^{2+} and or EDTA can be used to construct IMPLICATION (IMP) and INHIBIT (INH) logic functions (Fig. 7). An IMP logic gate was constructed by monitoring absorbance at 410 nm and with two inputs (EDTA and Cu^{2+}). The absorbance at 410 nm was low (off state) than that of threshold value only in the presence of Cu^{2+} (0, 1). However, the high absorbance (on state) was observed in the absence (0, 0) and presence (1, 1) of both inputs and also EDTA alone (Table 2). An INH logic gate, demonstrating noncumulative behavior was constructed by using similar chemical inputs Cu^{2+} and EDTA, and absorbance at 450 nm as an output signal. In the presence of Cu^{2+} alone (1, 0), the absorbance at 450 nm was high enough (on state). In the presence of EDTA (0, 1) or both (1, 1), the absorbance was low (off state).

3.2.6. Fluorogenic Sensing of Cu^{2+} Ions

Since we are interested in the detection of Cu^{2+} ions in aqueous solution thus all the fluorescence studies was further conducted in ethanol–buffer solution (1:1, v/v, HEPES 10 mM, pH = 7.4) only. In order to understand the binding interactions of Schiff base 1 with Cu^{2+}, fluorescence titration experiments were performed at r.t. (Fig. 8). The fluorescence intensity at both the wavelength decreases with concomitant addition of Cu^{2+} ions (0–27.7 μM) in 10 μM Schiff base 1, and almost complete quenching was observed upon addition of 2.7 equiv. of Cu^{2+}. The concurrent decrease in intensity at both emission bands also proofs the existence of single species in solution. Fluorescence is quenched because the presence of Cu^{2+} ions which have empty d orbital (d^9), leads to faster forbidden intersystem crossing (ISC) from S_1 to T_1 state of fluorophore, that is deactivated by bimolecular non–radiative processes (paramagnetic effect).

Fluorescence quenching mechanism may be static or dynamic type. Static quenching corresponds to change in UV–Vis spectra of host in the presence of guest molecule. In the case of Schiff base 1, addition of Cu^{2+} ions results in appearance of a new band, as discussed earlier, validating

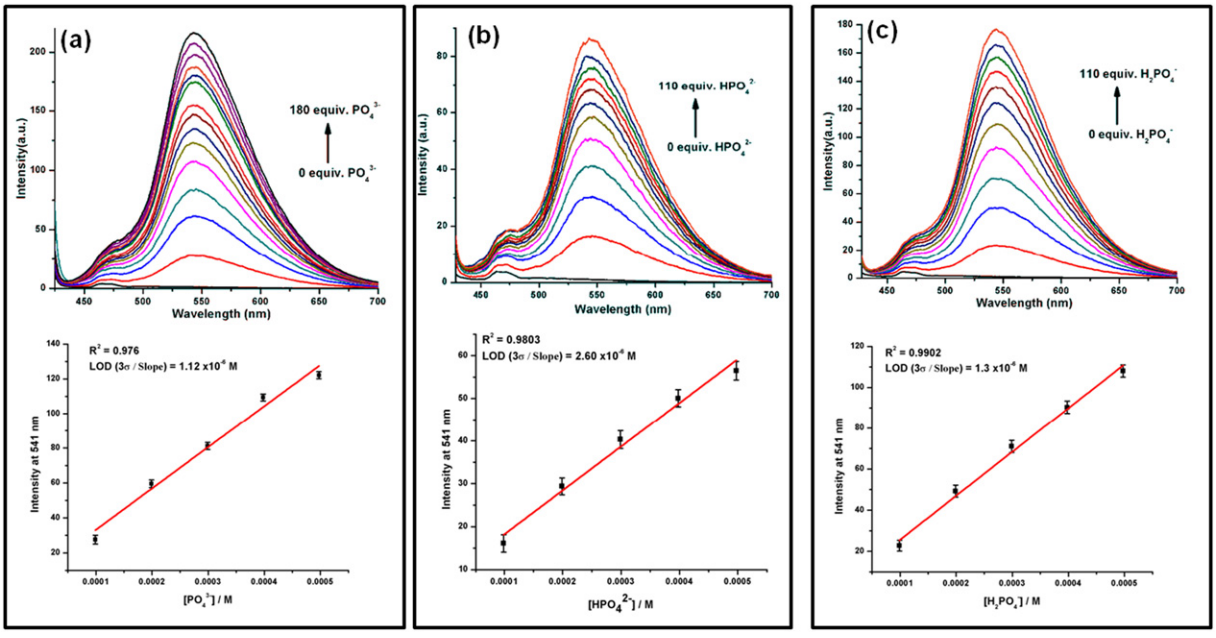


Fig. 10. Changes in fluorescence intensity of mixture solution, 10 μM Schiff base 1 + 10 equiv. Cu^{2+} upon addition of different phosphate ions, PO_4^{3-} (a), HPO_4^{2-} (b) and $H_2PO_4^-$ (c), and their corresponding detection limit curve plot. The error bars represent the standard deviation of three independent measurements.

the static type quenching. The UV–Vis changes evidenced formation of 1 − Cu^{2+} ground state complex, so it is concluded that the fluorescence quenching of Schiff base 1 induced by Cu^{2+} ions is primarily due to the complex formation. Quenching constant 1.8×10^5 L Mol^{-1} was calculated by the use of Stern–Volmer equation (Fig. S13). The LOD of Schiff base 1 for Cu^{2+} from fluorescence titration curve was determined to be 3.4×10^{-7} M, indicating high detection sensitivity (Fig. 8). Binding number of Cu^{2+} with Schiff base 1 was obtained by using double–logarithmic equation. Log [I_o–I/I] is a function of log [Cu^{2+}], a straight line is obtained with slope n (binding number). From this linear regression plot ($R^2 = 0.9979$), value of n is found to be very close to 1, indicating 1:1 stoichiometry between Schiff base 1 and Cu^{2+}. Simultaneously, association constant, log K_b (5.92) was calculated by using the same equation indicating high binding affinity between Schiff base 1 and Cu^{2+} (Fig. S13). Furthermore, competition experiments were also performed for Schiff base 1 at 10 μM in the presence of 100 μM of Cu^{2+} with 10 equiv. of aforementioned ions in ethanol–buffer solution (1:1, v/v, HEPES 10 mM, pH = 7.4) (Fig. 9). Although, the various ions did not show significant changes in the fluorescence intensity of 1 − Cu^{2+} complex, however, the presence of 10 equiv. of phosphate ions (PO_4^{3-}, HPO_4^{2-} and $H_2PO_4^-$) causes fluorescence enhancement at 537 and 464 nm bands.

3.3. Fluorogenic Sensing of Phosphate Ions

The above results indicate that 1 − Cu^{2+} complex could be used as a receptor for the sensing of phosphate ions. The 1 − Cu^{2+} was found to unable to distinguish between three types of tested phosphate ions at physiological pH. Upon addition of incremental concentration of PO_4^{3-} (0–180 equiv.), HPO_4^{2-} (0–110 equiv.) and $H_2PO_4^-$ (0–110 equiv.) in ethanol–buffer solution (1:1, v/v, HEPES 10 mM, pH = 7.4) of 1 − Cu^{2+} (10 μM + 100 μM Cu^{2+}), the fluorescence intensity increases gradually (Fig. 10). From the corresponding fluorescence titration profile LOD for PO_4^{3-}, HPO_4^{2-} and $H_2PO_4^-$ were calculated to be 1.1 μM, 2.6 μM and 1.3 μM respectively (Fig. 10). The fluorescence recovery may be due to the release of Schiff base 1 from 1 − Cu^{2+} complex owing to chelation of phosphate with Cu^{2+}. Binding constants (logK) for phosphate ions was calculated by applying Eq. (1) and was found to be 4.59, 5.19 and 5.58 for PO_4^{3-}, HPO_4^{2-} and $H_2PO_4^-$ respectively. Other anions do not show significant fluorescence enhancement

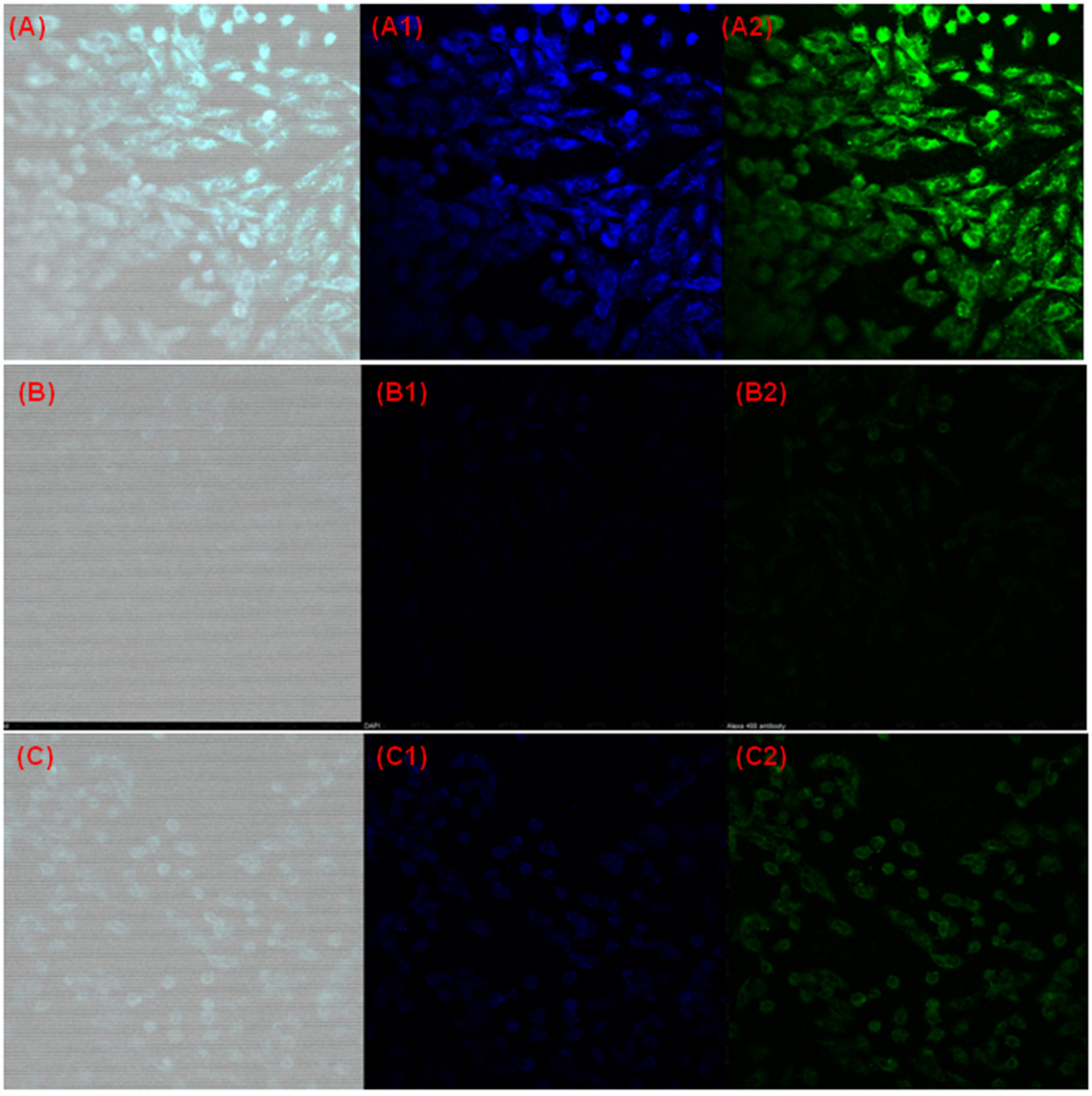


Fig. 11. Living cell imaging, bright field image (A) and fluorescence images (A1 and A2) of A549 cells incubated with Schiff base 1. bright field image (B) and fluorescence images (B1 and B2) of A549 cells incubated with Schiff base 1 and Cu^{2+}. Bright field image (C) and fluorescence images of A549 cells incubated with Schiff base 1 + Cu^{2+} + PO_4^{3-}.

comparing with phosphate may be because of high basicity of phosphate ions in aqueous solution. The competition experiment in the presence of other ions Co^{2+}, Ni^{2+}, Zn^{2+}, Br^-, Cl^- and F^- was performed by taking PO_4^{3-} as representative among other phosphate ions and found insignificant effect in the detection of PO_4^{3-}.

3.4. Live Cell Imaging

The cytotoxicity of Schiff base 1 on A549 cells was analyzed by performing MTT assay at different concentrations. Schiff base 1 did not show any significant toxicity at the tested concentrations. Thus, it can be used as a sensor to detect Cu^{2+} and phosphate ions in living cells.

In order to evaluate the real practical application of Schiff base 1 for the detection of Cu^{2+} and phosphate ions (PO_4^{3-}), live cell imaging experiments were performed in A549 cells. A549 cells incubated with 5 μM Schiff base 1 showed bright fluorescence in both blue and green channel as shown in Fig. 11. However, when A549 cells, pre-treated with Schiff base 1 was incubated with 20 μM Cu^{2+}, the fluorescence was found to quenched significantly. Further, addition of 200 μM PO_4^{3-} ions to A549 cells, pre-treated with Schiff base 1 + Cu^{2+} restore the fluorescence. These results clearly specify the biological application of Schiff base 1 to detect intracellular Cu^{2+} and PO_4^{3-} ions by fluorescence microscopy.

4. Conclusions

In conclusion, an unsymmetrical azine based Schiff base, 5-diethylamino-2-[(2-hydroxy-benzylidene)hydrazonomethyl]-phenol (1) was synthesized by simple condensation reactions with excellent yield. Schiff base 1 has been utilized for the chromo–fluorogenic sensing of Cu^{2+} ions in ethanol–buffer solution (1:1, v/v, HEPES 10 mM, pH = 7.4) and its 1 − Cu^{2+} complex for fluorogenic sensing of phosphate ions (PO_4^{3-}, HPO_4^{2-} and $H_2PO_4^-$) in the same above mentioned aqueous solution. The present Schiff base 1 shows interesting aspects over existing azine based Schiff bases, viz. (i) absence of aggregation induced emission (AIE) (ii) strong fluorescence in both organic and aqueous solution (iii) ESIPT fluorescence (ICT is suppressed) (iv) ion sensing behavior is investigated in competitive protic solvents (v) least interference by other ions for the detection of Cu^{2+} ions (vi) biological application for detection of Cu^{2+} and PO_4^{3-} ions in living cells.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.saa.2017.09.072.

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