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RESEARCH ARTICLE

Carbazole–azine based fluorescence '*off–on*' sensor for selective detection of Cu^{2+} and its live cell imaging

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Abstract

A new carbazole–azine based fluorescent sensor was synthesized and characterized. The selectivity of the sensor for Cu^{2+} over other counter ions in a dimethyl sulfoxide/H_2O mixture was shown through enhancement in fluorescence – an *off* to *on* transformation. The specificity of the probe towards Cu^{2+} was evident in ultraviolet/visible, fluorescence, Fourier transform infrared and mass studies. Application of the probe in the cell imaging and cytotoxicity of living cells is illustrated.

KEYWORDS

azine, carbazole, cell imaging, copper ion, fluorescence

1 | INTRODUCTION

The design and synthesis of new chemosensors for monitoring ionic species, especially heavy and transition metal ions, are of great interest in chemical, biological and environmental analyses.[1–9] Copper is an essential trace element in biological systems, although under overdose conditions, it causes neurodegenerative diseases (e.g. Alzheimer's and Wilson's diseases).[10–16] The toxicity of copper ions towards humans is rather low compared with that of other heavy metals, but certain microorganisms are affected by low concentrations of copper.[17] The United States Environmental Protection Agency recommends an upper limit of 1.3 mg copper in humans.[18] Because of the toxicity of copper ions and their role as a critical catalytic co-factor in a variety of metallo enzymes, such as superoxide dismutase, cytochrome *c* oxidase and tyrosinasemore, researchers have explored efficient recognition probes for Cu^{2+}.[19–21] Although many analytical techniques have been developed, they are time consuming and expensive, and a simple, distinct reaction of toxic metal ions via colorimetric quantification is required. Thus, the development of fluorescence-based sensors for metal ions has attracted much interest among the research community.[22–27]

Cu^{2+} is well known among transition metals as a fluorescent quencher due to its paramagnetic nature. It is therefore practically difficult to develop fluorescent sensors for the detection of Cu^{2+}.[28–32] In recent years, there have been several attempts to design chemosensors for paramagnetic metal ions. Recently, rhodamine B-based fluorescent probes have been reported as 'turn-on' fluorescence, and colorimetric, sensors for Cu^{2+}.[33–42] In these sensors, the mechanism involves opening of the spirolactam ring upon binding with a Cu^{2+} ion, resulting in fluorescence enhancement. In addition, several other chemosensors have been reported with different signaling mechanisms for the optical detection of paramagnetic ions based on photo-induced electron transfer, metal–ligand charge transfer, intramolecular charge transfer (ICT), excimer/exciplex formation, and excited-state intermolecular proton transfer.[43–58]

Carbazole and its derivatives possess highly conjugated systems, engage in active ICT and are used as electron-donating chromophores.[59–63] Carbazole compounds are promising candidates for electroluminescent and photorefractive devices because of their electron-donating and hole-transporting properties, and their remarkable application as fluorescent sensors.[64–76] In the bis-azine type ligand, the electron density is located on the [C=N–N=C] moiety. Binding

Abbreviations used: **$[CzA]_2$-Cu**, Carbazole–azine–copper complex; **CzA**, Carbazole–azine based sensor (N,N′-bis(9-ethyl 3-carbazolylmethylidene) azine); DFT, Density functional theory; DMSO, Dimethyl sulfoxide; ESI-MS, Electrospray ionization mass spectrometry; FTIR, Fourier transform infrared spectroscopy.; HOMO, Higher occupied molecular orbital; ICT, Intramolecular charge transfer; LUMO, Lower unoccupied molecular orbital; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NMR, Nuclear magnetic resonance spectroscopy; PBS, Phosphate-buffered saline; TDDFT, Time-dependent density functional theory; UV, Ultraviolet; Vis, Visible

of metal ions to the ligand affects the electron density and thus influences the fluorescence of the ligand.[77–82] In this work, we developed a new carbazole–azine probe, **CzA**, as a sensor for Cu^{2+} in a dimethyl sulfoxide (DMSO)/H_2O mixture via enhancement of the fluorescence – an off to on transformation.

2 | EXPERIMENTAL

2.1 | Materials and reagents

Starting materials and reagents such as *N*-ethyl carbazole, dimethyl formamide, phosphorous oxy chloride and hydrazine monohydrate were purchased from Sigma-Aldrich Co. Ltd. (India) and used as received. Solvents used for synthesis and extraction were obtained from Sisco Research Laboratories Pvt. Ltd. (India), and were distilled and dried according to standard procedures before use. Spectroscopic grade solvents from Central Drug House Ltd. (India) were used for photophysical studies.

2.2 | Measurements

Melting point was determined using a hot-plate melting point apparatus in an open mouth capillary and is uncorrected. ^{1}H NMR spectra were recorded at 300 MHz on a Bruker FT–NMR spectrometer in DMSO-d_6 with tetramethylsilane as the internal standard. ^{13}C NMR spectra were recorded at 400 MHz on a Bruker FT–NMR spectrometer in DMSO-d_6 with tetramethylsilane as the internal standard; any chemical shift was measured in ppm. ESI-MS analysis was performed in positive ion mode on a liquid chromatography-ion trap mass spectrometer (LCQ Fleet, Thermo Fisher Instruments Ltd, USA). FTIR spectra were recorded with a Perkin–Elmer spectrometer equipment (version 10.03.09) using KBr pellets. UV/Vis and fluorescence spectroscopy measurements were carried out using a Shimadzu (model UV1700) spectrophotometer and spectrofluorimeter (model FP-8200), respectively, with a quartz cell of path length 10 mm. Fluorescent quantum yields were determined using Horiba-FluoroMax 4 spectrophotometer. Fluorescence imaging was performed using a Nikon confocal fluorescence microscope. The syntheses were dry reactions, with magnetic stirring, performed under a nitrogen gas atmosphere. The reactions were constantly monitored using thin-layer chromatography. Metal solutions were prepared from their nitrate salts. Milli-Q water was used throughout the analytical experiments.

2.3 | MTT assay

Cytotoxicity towards and the cell viability of HeLa cells were tested using the MTT assay procedure by reduction of MTT to formazan crystals. The cells and probe **CzA** were prepared in 96-well plates, with 10 µl MTT solution added, and incubated at 37°C for 4 h. DMSO (100 µl) was added to the formed formazan crystals in each well to dissolve the crystals, the plates were shaken for 10 min. The absorbance was recorded at 570 and 630 nm. Percent cell viability was determined from the formula: % survival = [live cell number (test)/live cell number (control)] × 100.

2.4 | Cell culture and fluorescence imaging

HeLa cells were cultured as a monolayer in Eagle's minimum essential medium, maintained at 37°C and supplemented with 10% fetal bovine serum. Cells were treated with **CzA** (5 µM) in DMSO: PBS buffer and incubated at pH 7.54 for 30 min. Thereafter, the cells are washed three times with PBS to remove any excess probe, followed by incubation with $CuCl_2$ (10 µM in DMSO) for 10 min at 37°C. Cells were monitored using Nikon fluorescence microscopy.

2.5 | Synthesis

2.5.1 | Precursor 9-ethyl-9H-carbazole-3-carbaldehyde (2)

Precursor **2** was synthesized in accordance with formerly reported methods (Supporting Information).

2.5.2 | *N*,*N*′-bis(9-ethyl 3-carbazolylmethylidene)azine (CzA)[83–85]

Hydrazine monohydrate (0.089 g, 1.79 mmol) was added slowly with stirring to a solution of precursor **2** (0.4 g, 17.9 mmol) in 40 ml methanol. The resulting mixture was refluxed at 65°C for 12 h under a nitrogen atmosphere. The mixture was cooled to room temperature, and the yellow solid appeared was filtered, washed with MeOH and Et_2O, and dried in a vacuum. *N*,*N*′-bis(9-ethyl 3-carbazolylmethylidene)azine (**CzA**), yellow solid, yield: 90%; m.p.: 143°C; FTIR (KBr) cm^{-1}: 3050.83 (=C-H), 2969.84, 2923.56, 2859.10 (N–C), 1734.66, 1607.38 (C–C monosubstituted phenyl), 1469.49 (C = C conjugated phenyl group), 1340.28 (–C–N_{str}), 1232.29, 1131.05, 1024.02 (–HC = N, out of plane bending vibration), 885.166, 808.992 (C–H, *p*-substituted benzene ring), 741.496, 667.25, 600.717 (C–H phenyl rings). ^{1}H NMR (DMSO-d_6, 300 MHz), δ_H (ppm): 1.33 (4H, t, *J* = 6.9 Hz); 4.49 (2H, q, *J* = 7.2 Hz); 7.26 (4H, t, *J* = 7.5 Hz); 7.50 (4H, t, *J* = 7.5 Hz); 7.65–7.74 (4H, m); 8.04 (2H, d, *J* = 8.7 Hz); 8.23 (2H, d, *J* = 7.8 Hz); 8.65 (2H, s); 8.90 (2H, s). ^{13}C NMR (DMSO-d_6, 400 MHz), δ_C (ppm): 161.96, 141.73, 140.55, 126.83, 126.25, 125.54, 122.91, 122.65, 121.90, 121.12, 120.05, 110.10, 37.70, 14.23. Mass (ESI-MS positive mode): 442.6766 (Supporting Information).

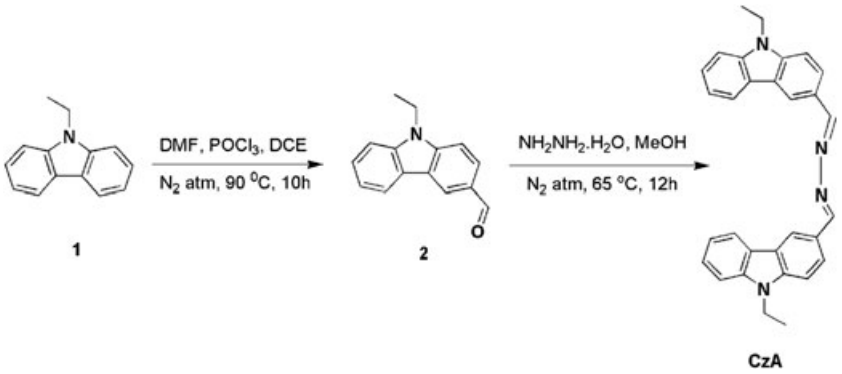
3 | RESULT AND DISCUSSION

3.1 | Synthesis

The synthesis of **CzA** is shown in Scheme 1. Initially, compound **2** was synthesized by Vilsmeier formylation of *N*-ethyl carbazole (**1**). Probe **CzA** was then prepared by refluxing compound **2** with hydrazine monohydrate in the presence of MeOH. The crude products of **2** and **CzA** were purified by column chromatography and dried. The purity of these compounds was established using FTIR, ^{1}H NMR, ^{13}C NMR and ESI-MS (Fig. S1–S4).

3.2 | UV/Vis spectral studies

UV/Vis titration was undertaken to establish the specificity and the nature of the association of **CzA** towards Cu^{2+} ion (Fig. 1). The certainty of **CzA** binding to Cu^{2+} was shown by competitive absorption



SCHEME 1 Synthesis of probe **CzA**

studies with variable metal ions in DMSO: H_2O (8: 2), where the other metal ions, Na^+, K^+, Ag^+, Mn^{2+}, Cd^{2+}, Cu^{2+}, Fe^{3+}, Al^{3+}, Cr^{3+}, Pb^{2+}, Co^{2+}, Mg^{2+}, Zn^{2+} and Ni^{2+}, do not have any significant effect on the absorbance. Probe **CzA** showed strong absorption bands at λ_{max} = 240, 298, 352 and 372 nm on addition of a solution of Cu^{2+} ions. A hyperchromic shift in the absorbance was noticed, despite the other competing metal ions (Fig. S5). Comparatively, on titration of **CzA** (10 μM) with a Cu^{2+} ion solution ranging from 0 to 1 equiv. Cu^{2+} (10 μM) in the same solvent composition, a simultaneous increase in intensity was evident. These observations suggest that the response of probe **CzA** is highly Cu^{2+} specific with prominent enhancement in the absorbance.

3.3 | Fluorescence spectral studies

Emission spectral analysis of probe **CzA** in countering various metal ions in DMSO: H_2O (8: 2) was undertaken. The study shows that weak fluorescent probe **CzA** (10 μM) resulted in emission enhancement at 556 nm by binding with Cu^{2+} on excitation at λ_{ex} = 372 nm (Fig. 2). There was no apparent deviation in the emission spectra of other metal ions on encountering Cu^{2+} ion (Fig. S6). This fluorescence study shows a 2: 1 stoichiometry, and selectivity towards Cu^{2+} in the presence of interfering metal ions. The results coincide with the interference seen in the absorption studies.

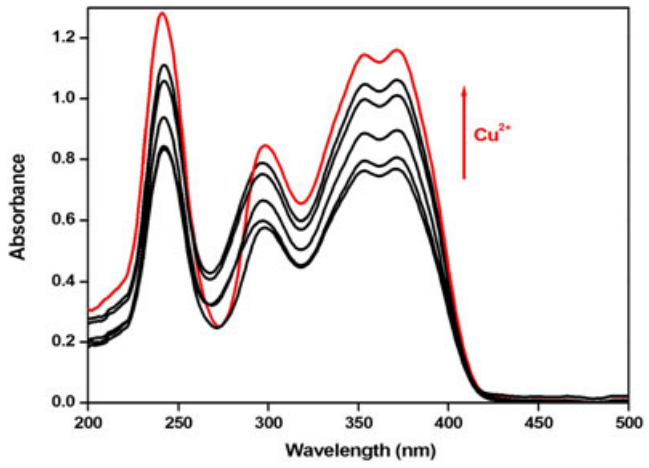


FIGURE 1 UV/Vis spectrum of probe **CzA** (10 μM) upon addition of Cu^{2+} (0–1 equiv.) in a DMSO: H_2O (8: 2) mixture

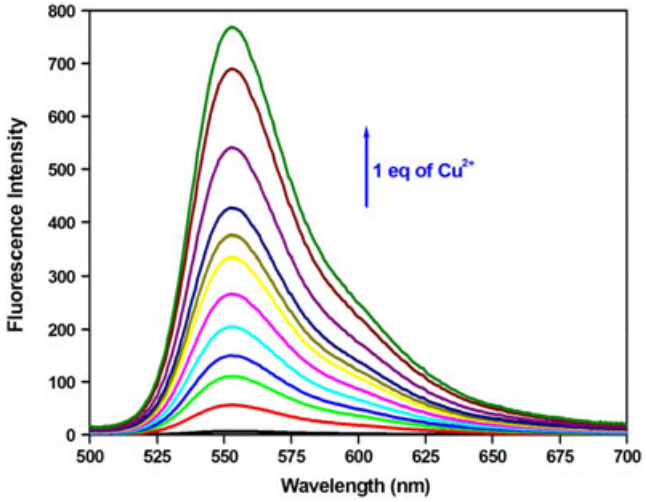


FIGURE 2 Fluorescence emission spectrum of probe **CzA** (10 μM) upon addition of Cu^{2+} (λ_{ex} = 372 nm) (0–1 equiv.) in a DMSO: H_2O (8: 2) mixture

3.4 | Job's plots

To determine the stoichiometric binding ratio of probe **CzA** with Cu^{2+} ion, Job's plot analysis was undertaken. A plot of the absorbance at 372 nm over mole fraction was linear with maxima at 0.6 mole fractions of Cu^{2+} ion, which indicates a 2: 1 stoichiometry; this was confirmed by the peak at 946.2936 **$[CzA]_2$–Cu^{2+}** in the ESI-MS analysis (Fig. S8). Jobs plots from the fluorescence analysis were also in agreement with the former result (Fig. S7). The association constant for the complex of **CzA** with Cu^{2+} was 2.2×10^4 M^{-1}.[86]

Emission titration of the probe with Cu^{2+} ions showed a progressive increase in fluorescence with increasing amounts of Cu^{2+}. After addition of 1.2 equiv. Cu^{2+} ion, the mixture reaches saturation point. In practice, the quantitative detection limit determined from the fluorescence spectral changes varies linearly with the Cu^{2+} concentration and was found to have a lower limit of 3.5×10^{-8} M.[87]

Fluorescence recognition by the probe of other metal ions competing with Cu^{2+} was investigated (Fig. 3). The probe showed no distinguishable change in fluorescence towards all other metal ions in the presence of Cu^{2+}, which confirms the ability of the probe to retain its specificity for Cu^{2+} ion in a pool of distinct metal ions. The quantum yields of the probe and the complex were calculated using a Horiba-FluoroMax 4 spectrophotometer and found to be 0.02 for probe

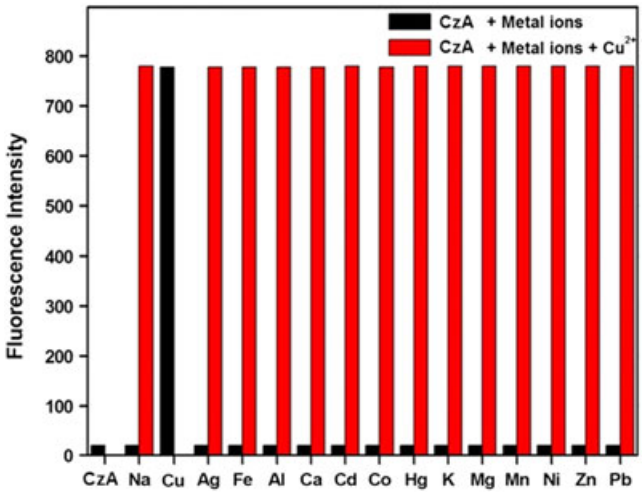


FIGURE 3 Fluorescence response of 10 μM **CzA** with various metal ions. Black bars represent the addition of the corresponding metal ion to **CzA**. Red bars represent the change of the emission that occurs upon the subsequent addition of Cu^{2+} to the above solution

CzA; a much higher quantum yield of 0.51 was observed for **CzA** upon addition of Cu^{2+} ion, which might be due to ICT. The electron density in the azine unit is located on the [C=N–N=C] moiety, binding of metal ions to the ligand affects the electron density and thus influences the fluorescence of the ligand. All the observed photophysical properties confirm the capability of **CzA** for the quantitative detection Cu^{2+}, an 'off–on'-type probe.

3.5 | Factors influencing fluorescence detection

The probe acts as a rapid fluorescence detector for copper ions. On prolonging the reaction time to 0.2 min, the emission becomes brighter, with a sharp rise in intensity. The intensity continues to increase with further increases in the reaction time to 1 min; becoming constant thereafter (Fig. S9). The effect of pH on the fluorescent detection of Cu^{2+} was tested. Under highly acidic conditions, the fluorescence is high, but decreases as the pH of the solution is increased, becoming very low and constant at pH 5 and beyond (Fig. S10). In addition, the reversibility of binding between the Cu^{2+} ion and the probe **CzA** was tested. It was found that EDTA is a proficient reagent for the reversibility, with fluorescent *on* to *off* signaling (Fig. S11).

3.6 | FTIR studies

The above results were further supported by FTIR (Fig. S12). Here, the stretching frequency of the [N–C] vibration of the **CzA** probe shifts on binding with copper ions from 2969.84, 2927.70 and 2859.10 cm^{-1} to 2965.98, 2923.56 and 2852.93 cm^{-1} respectively. The value of the [–HC=N] out of plane bending vibration, 1232.29, 1131.05 and 1024.02 cm^{-1}, shifts slightly to 1261.22, 1095.37 and 1025.94 cm^{-1}, respectively, due to ICT towards the Cu^{2+} ion via lone pair electrons in the nitrogen atom of the [C=N–N=C] entity. Because of the strong electron-donating capacity and ICT ability of the carbazole unit towards the azine moiety, the band for [=C–H] of the [C=N–N=C] moiety in **CzA** at 3060.83 cm^{-1} is not present for the **$[CzA]_2$–Cu** complex. In addition, the stretching frequency of the [C–C] mono-substituted phenyl entity exhibits a shift from 1607.38 to 1618.96 cm^{-1}. The intense band at 3434.6 cm^{-1} in **$[CzA]_2$–Cu** is characteristic of OH_{str}, which shows the involvement of water molecules in the binding mechanism, suggesting a tetrahedral coordinate system for the complex; something similar was also observed for the mass spectra of the complex (Fig. S11). A plausible binding mechanism for **CzA** towards Cu^{2+} is shown in Scheme 2.

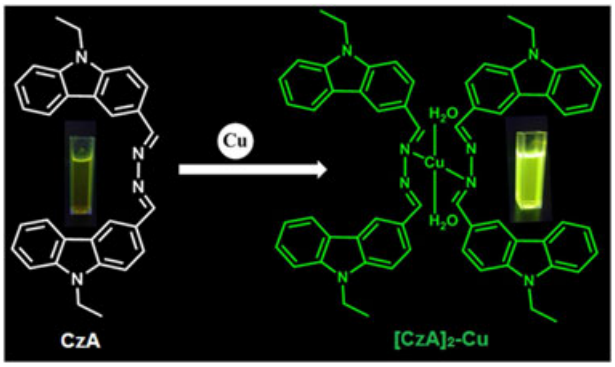
3.7 | Cell imaging and cytotoxicity studies

A cell viability assay was used to study the cytotoxicity of the probe **CzA** towards HeLa cells in DMSO at different doses and times. The assay revealed that the probe is less toxic to cells and has no apparent effect on cell viability; the solvent also had no effect on the cells. By contrast, **CzA** + Cu2^{2+} complex led to an eventual reduction in the cell viability of HeLa cells at concentrations >20 μM. This dose-dependent cytotoxicity was greater at higher concentrations and resulted in reduced cell viability. These observations are in accordance with previous reports, providing support for use of the **CzA** + Cu^{2+} complex on cancer cells (Fig. S13 and S14).[88]

The cytotoxic assay results suggested 10 μM **CzA** + Cu^{2+} as optimal for use in cell imaging studies in HeLa cells. To measure the ability of the probe to detect Cu^{2+} ions in live cells using confocal microscopy, MTT-mediated HeLa cells were incubated with **CzA** (5 μM) in DMSO for 30 min at 37°C produce a 'off' fluorescent image of the cell. The cells were then treated with Cu^{2+} solution for 10 min at the same temperature; this led to a 'switch on' green fluorescence in the HeLa cells (Fig. 4), indicating the formation of the **CzA** + Cu^{2+} complex in the cell, as seen in the optical studies. These observations suggest use of the **CzA** probe for the non-cytotoxic, biocompatible and spontaneous detection of Cu^{2+} ion in living cells.

3.8 | Density functional theory calculations

To confirm the above results, density functional theory (DFT) studies were undertaken to provide a theoretical basis for **CzA** sensor behavior and selectivity towards Cu^{2+} ion. The DFT calculation was performed using the Gaussian 09 program (B3LYP and 6-311G/LANL2DZ basis).[89] The optimized geometry of the probe **CzA** and **CzA** + Cu^{2+} confirmed 2: 1 stoichiometric binding of **CzA** to Cu^{2+} ion through [N–Cu–N] binding (Fig. S15). The TDDFT calculation was carried out



SCHEME 2 Proposed binding mechanism of **CzA** towards Cu^{2+}

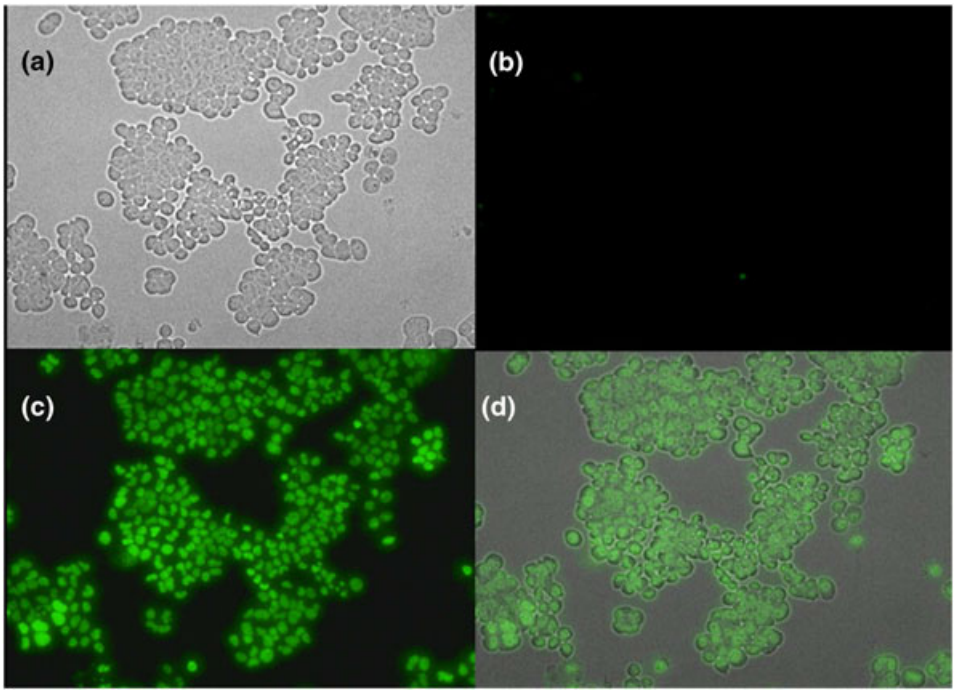


FIGURE 4 (a) bright field image of HeLa cells. (b) fluorescence image of HeLa cells incubated with **CzA** (10 μM) for 2 h at 37°C. (c) fluorescence image of **CzA** treated HeLa cells with Cu^{2+} (10 μM). (d) bright field images of HeLa cells incubated with **CzA** + Cu^{2+}

from optimized figures. In the frontier molecular orbitals of probe **CzA**, the HOMO spread over the π-cloud of the carbazole fragment and the LUMO was mounted on the azine unit. In the frontier molecular orbitals of complex **CzA** + Cu^{2+}, the HOMO was partly localized on the π-cloud of carbazole and azine, whereas the LUMO was towards the Cu^{2+} ion. The energy gap of **CzA** + Cu^{2+} was lower than that of **CzA** (Fig. 5), which promotes easy transition. Therefore, from the DFT calculation, it is clear that the probe and the complex **CzA** + Cu^{2+} are involved

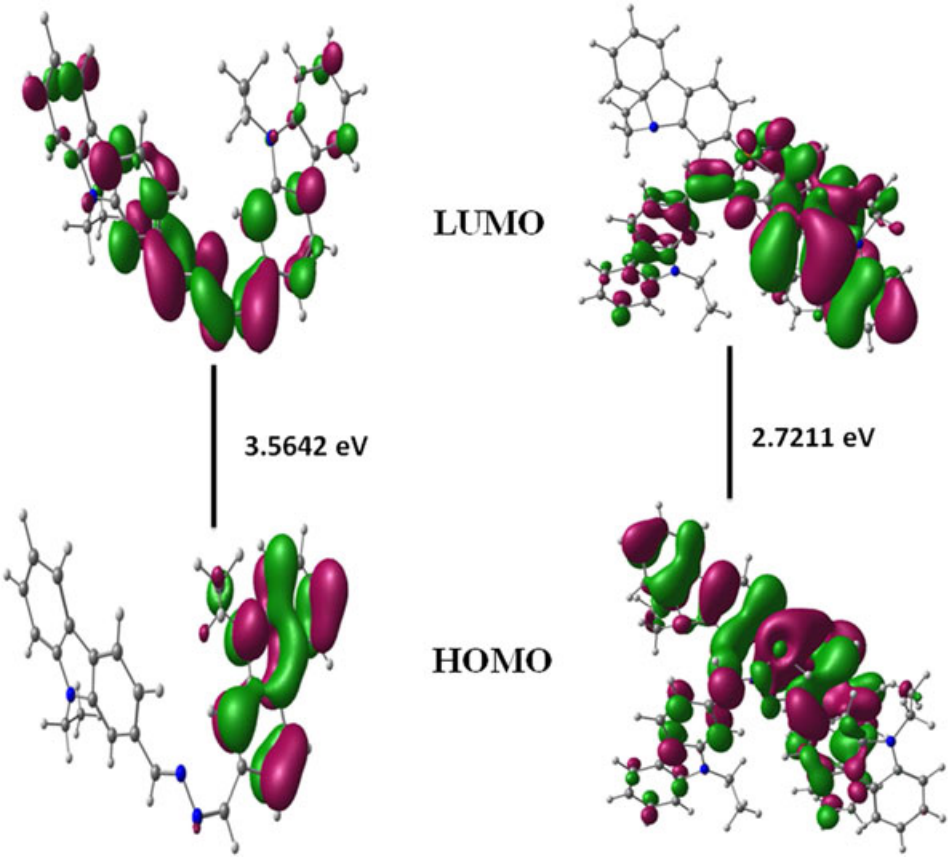


FIGURE 5 Calculated (B3LYP/6-31G*) structure for **CzA** and **CzA** + Cu^{2+} complex

in π–π* transition from HOMO to LUMO, with greater opportunity for ICT to govern probe **CzA** on approaching Cu^{2+} ion.

4 | CONCLUSION

We have designed and synthesized a carbazole-based azine derivative **CzA** probe that was shown to be a highly selective, sensitive sensor for Cu^{2+} ion. The sensitivity of probe **CzA** for Cu^{2+} was demonstrated in living cells, and a cell toxicity assay revealed that **CzA** can be used for selective imaging of Cu^{2+} ions in living cells.

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