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Visible–Near-Infrared and Fluorescent Copper Sensors Based on Julolidine Conjugates: Selective Detection and Fluorescence Imaging in Living Cells

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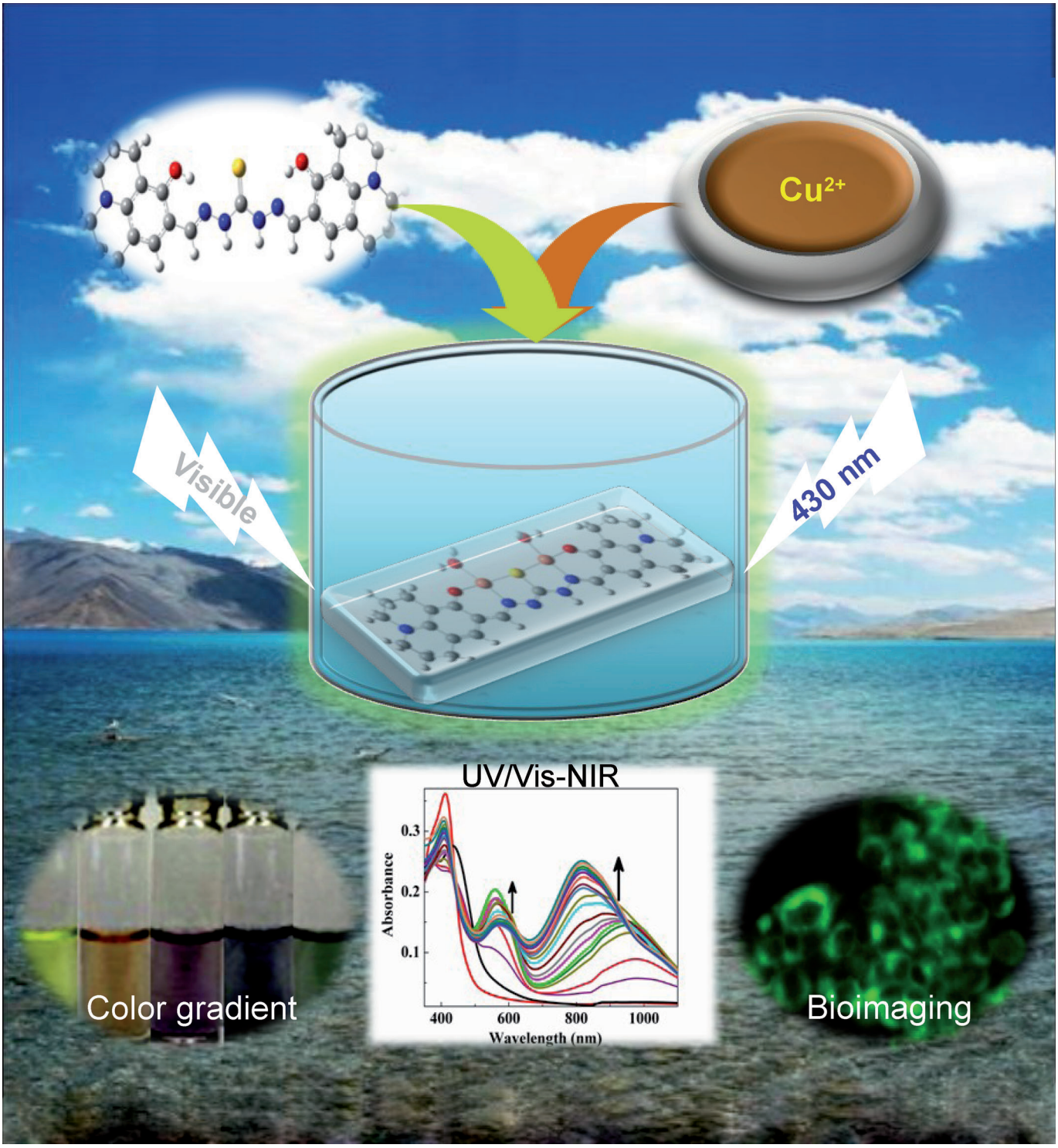

Abstract: We present novel Schiff base ligands julolidine–carbonohydrazone **1** and julolidine–thiocarbonohydrazone **2** for selective detection of Cu^{2+} in aqueous medium. The planar julolidine-based ligands can sense Cu^{2+} colorimetrically with characteristic absorbance in the near-infrared (NIR, 700–1000 nm) region. Employing molecular probes **1** and **2** for detection of Cu^{2+} not only allowed detection by the naked eye, but also detection of varying micromolar concentrations of Cu^{2+} due to the appearance of distinct coloration. Moreover, Cu^{2+} selectively quenches the fluorescence of julolidine–thiocarbonohydrazone **2** among all other metal ions, which increases the sensitivity of the probe. Furthermore, quenched fluorescence of the ligand **2** in the presence of Cu^{2+} was restored by adjusting the complexation ability of the ligand. Hence, by treatment with ethylenediaminetetraacetic acid (EDTA), thus enabling reversibility and dual-check signaling, julolidine–thiocarbonohydrazone (**2**) can be used as a fluorescent molecular probe for the sensitive detection of Cu^{2+} in biological systems. The ligands **1** and **2** can be utilized to monitor Cu^{2+} in aqueous solution over a wide pH range. We have investigated the structural, electronic, and optical properties of the ligands using ab initio density functional theory (DFT) combined with time-dependent density functional theory (TDDFT) calculations. The observed absorption band in the NIR region is attributed to the formation of a charge-transfer complex between Cu^{2+} and the ligand. The fluorescence-quenching behavior can be accounted for primarily due to the excited-state ligand **2** to metal (Cu^{2+}) charge-transfer (LMCT) processes. Thus, experimentally observed characteristic NIR and fluorescence optical responses of the ligands upon binding to Cu^{2+} are well supported by the theoretical calculations. Subsequently, we have employed julolidine–thiocarbonohydrazone **2** for reversible fluorescence sensing of intracellular Cu^{2+} in cultured HEK293T cells.

Keywords: bioimaging · chemosensors · copper · fluorescence · visible–near infrared

Introduction

Molecular sensors have been developed for selective recognition of different species on the basis of host–guest interactions making use of hydrogen bonding, electrostatic force, metal–ligand coordination, and hydrophobic and van der Waals interactions.[1] In recent years, the development of novel colorimetric and fluorescent sensors of biologically active metal ions have been actively investigated because of their potential applications in life sciences, medicine, chemistry, and biotechnology.[2] The design and synthesis of highly selective sensors for metal ions, such as mercury, lead, iron, zinc, and copper, is particularly important, since these metal ions can have detrimental effects on the environment and human health.[3] Copper is one of a relatively small group of trace metal nutrients that are essential to sustain normal human health. However, copper has to be supplemented through human diet in regular amounts for absorption.[4] The adult human body contains between 1.4–2.1 mg of copper per kilogram of body weight under normal conditions. Copper-dependent enzymes act as catalysts to help a number of body functions to provide energy for biochemical reactions, transform melanin for pigmentation of the skin, assist the formation of cross-links in collagen and elastin, and thereby maintain and repair connective tissues. This is especially important for the heart and arteries[5] and research suggests that copper deficiency is one of the factors leading to an increased risk of developing coronary heart disease.[6] In general most human diets contain enough copper (2–5 mg day^{-1}) to prevent a deficiency, but not sufficient to cause toxicity. The World Health Organization (WHO) and the Food and Agricultural Administration (FAA) reports suggest that the population mean intake of copper should not exceed 10–12 mg day^{-1} for adults. Copper in excessive amounts can be toxic and cause oxidative stress and disorders associated with neurodegenerative diseases including Alzheimer's, Parkinson's, Menkes, Wilson's, and prion diseases.[7,8] Although protein and organically bound copper appears to be less toxic, free solvated Cu^{2+} is particularly damaging, since it catalyses the formation of reactive organic species (ROS), including radical and nonradical species, which can also trigger oxidative damage to proteins, nucleic acids, and lipids.[9] The common nutritional deficiencies of zinc, manganese, and other trace minerals also facilitate the accumulation of very high levels of copper.

In the past few years, many colorimetric and fluorescent chemosensors have been reported for sensing copper ions with absorbance and emission in the visible region.[10–18] Although chemosensors with visible (absorbance/emission) re-

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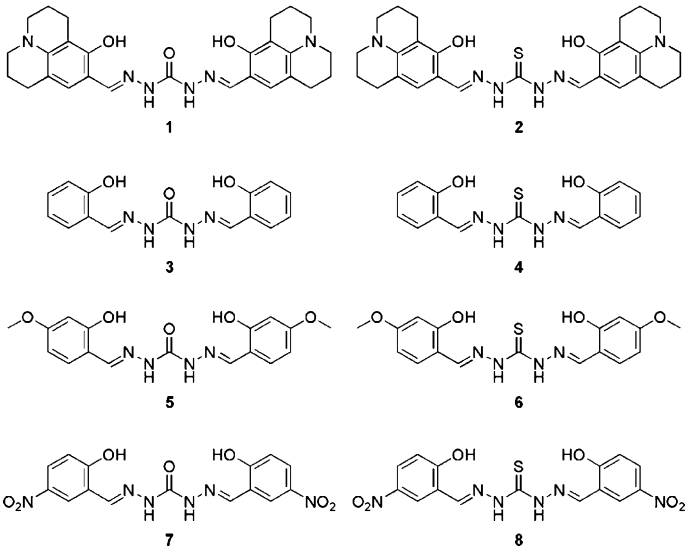
sponses have important roles in various research endeavors, molecular probes with near-infrared (NIR, 700–1000 nm) optical responses are particularly gaining special interest in recent years.[19] Unlike UV/Vis radiation, NIR radiation can penetrate much deeper into the sample, since there is no or limited absorption and scattering of NIR radiation. Additionally, there is no interference of autofluorescence generated from the chromophores and macromolecules present in the analytic samples. This phenomenon enables the assessment of molecular and physiological events in several layers deep inside the analyte samples and tissues.[20] There have been few reports on UV/Vis–NIR sensing of cation and anions.[21–24] Recently, we have reported Schiff base conjugates of urea/thiourea and salicylaldehyde as colorimetric chemosensors with characteristic NIR response for selective detection of copper.[25] Designing novel and efficient NIR chemosensors necessitates the need for understanding detailed molecular mechanism involved in the origin of such interesting phenomenon. The studies aimed at understanding the underlying mechanism and origin of NIR responses are scarce and hence theoretical calculations would be of great help to understand the microscopic details of such changes in the photophysical characteristics. Along with visible and NIR absorbance signals, a fluorescence response would add a new dimension to the sensing of metal ions over the methods involving just one kind of optical response. Additionally, the fluorescence chemosensors are known for their sensitivity, specificity, and real-time monitoring with fast response time.[26] Thus, designing a colorimetric chemosensor that combines the advantage of the characteristics of an NIR optical response with the sensitivity of fluorescence is of prime importance. In accordance with this concept we have designed two novel compounds julolidine–carbonohydrazone **1** and julolidine–thiocarbonohydrazone **2** for selective detection of Cu^{2+} by conjugating hydroxyjulolidinal with carbohydrazide and thiocarbohydrazide (Scheme 1). We have demonstrated their ability to detect Cu^{2+} colorimetrically with characteristic absorbance in the NIR region. Moreover julolidine–thiocarbonohydrazone **2** was used for the detection of Cu^{2+} fluorometrically, since it overall increases the sensitivity of the probe. Furthermore, we have demonstrated the utility of **2** for reversible bioimaging of Cu^{2+} in cultured living cells. The experimentally observed characteristic NIR optical response of ligands **1** and **2** in the presence of Cu^{2+} was supported by detailed theoretical calculations.



Scheme 1. Schiff base ligands synthesized by conjugating various aldehydes with carbohydrazide (**1**, **3**, **5**, and **7**) and thiocarbohydrazide (**2**, **4**, **6**, and **8**).

Results and Discussion

Synthesis of Schiff base ligands: We have synthesized a series of Schiff base ligands by a one-step condensation reaction. A solution of 8-hydroxyjulolidinal in ethanol was added slowly to a solution of carbohydrazide or thiocarbohydrazide in water. The reaction mixture was heated to reflux for 24 h with constant stirring. The reaction mixture was cooled to room temperature and the precipitate was filtered. The precipitate was washed with ethanol and dried under vacuum to obtain Schiff base ligands julolidine–carbonohydrazone **1** and julolidine–thiocarbonohydrazone **2**, respectively, in quantitative yield. Similarly, ligands **3**–**8** were prepared by condensing different salicylaldehyde derivatives with electron-donating and withdrawing functional groups with carbohydrazide or thiocarbohydrazide in good yields (Scheme 1). All the synthesized Schiff base ligands (**1**–**8**) were characterized by NMR spectroscopy, mass spectroscopy, and elemental analysis.

Absorbance study of Schiff base ligands with Cu^{2+} and other metal ions: We studied photophysical properties of different Schiff base ligands carrying electron-withdrawing and electron-donating functional groups. First, the photophysical properties of julolidinal-based Schiff base ligands **1** and **2** were investigated by monitoring the absorption spectral behavior upon addition of several metal ions such as Li^{+}, Na^{+}, Ba^{2+}, Sr^{2+}, Mg^{2+}, Al^{3+}, Ca^{2+}, Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Ag^{+}, Cd^{2+}, Hg^{2+}, Pb^{2+}, and Cu^{2+} in an aqueous buffer medium (50 mM, 2-(4-(2-hydroxyethyl)-1-piperazinyl)ethanesulfonic acid (HEPES)/CH_3CN, 6:4, v/v; pH 7.2). Ligands **1** and **2** exhibited characteristic absorbance in the visible (460–600 nm) and NIR (700–1100 nm) regions in the presence of Cu^{2+} as shown in Figures 1 and 2. The presence of other metal ions did not lead to the appearance of any such visible and NIR absorbance of ligands **1** and **2** (Figures 1 and 2). In contrast, other Schiff base ligands (**3**–**8**) did not exhibit any characteristic optical response in the presence of Cu^{2+} (see Figure S1 in the Supporting Information). Julolidine–carbonohydrazone **1** shows an absorption band centered around 380 nm, which remains unchanged upon addition of 50.0 equivalents of Li^{+}, Na^{+}, Ba^{2+}, Sr^{2+}, Mg^{2+}, Al^{3+}, Ca^{2+}, Mn^{2+}, Fe^{2+}, Ag^{+}, Cd^{2+}, and Pb^{2+}. With the addition of 50.0 equivalents of Co^{2+}, Ni^{2+}, Zn^{2+}, and Hg^{2+} the

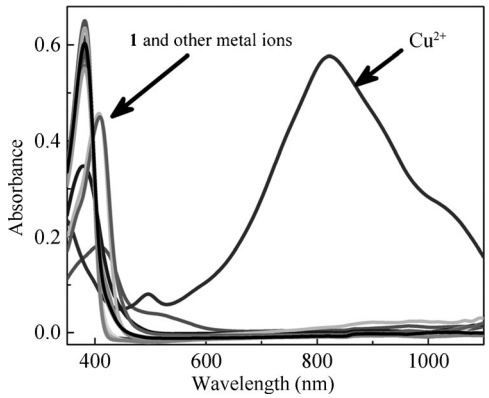


Figure 1. UV/Vis absorption spectra of julolidine–carbonohydrazone **1** (10.0 μM) and on addition of salts (50.0 equiv) of Li^+, Na^+, Ba^{2+}, Sr^{2+}, Mg^{2+}, Al^{3+}, Ca^{2+}, Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Ag^+, Cd^{2+}, Hg^{2+}, Pb^{2+}, and Cu^{2+} in aqueous medium (50 mM HEPES/CH_3CN, 6:4, v/v; pH 7.2).

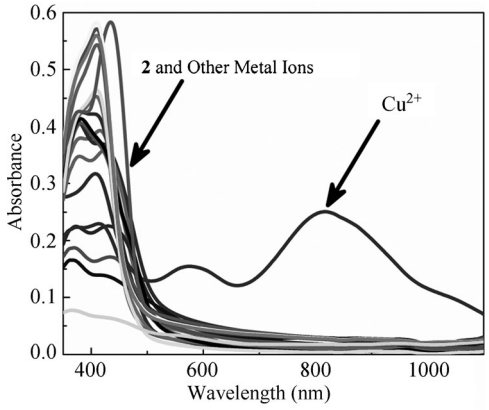


Figure 2. UV/Vis absorption spectra of julolidine–thiocarbonohydrazone **2** (10.0 μM) and on addition of salts (50.0 equiv) of Li^+, Na^+, Ba^{2+}, Sr^{2+}, Mg^{2+}, Al^{3+}, Ca^{2+}, Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Ag^+, Cd^{2+}, Hg^{2+}, Pb^{2+}, and Cu^{2+} in aqueous medium (50 mM HEPES/CH_3CN, 6:4, v/v; pH 7.2).

absorbance intensity decreases and is slightly red shifted to different extents as shown in Figure 1. At a similar concentration of Cu^{2+} (50.0 equiv), ligand **1** showed characteristic absorbance in the visible (495 nm) and NIR (823 nm) regions. During sequential titration, the absorption spectra of ligand **1** in the visible region gradually red shifted to 412 nm with the addition of 1.5 equivalents of Cu^{2+} and the colorless solution turns to light green. Increasing the concentration of Cu^{2+} in the solution of ligand **1** from 1.5 to 2.0 equivalents leads to a change in the solution color from light green to light purple. A new absorption band (λ_{max} = 570 nm) appeared in the visible region accompanied by a distinguishable NIR band with λ_{max} centered at 930 nm. The absorbance at 570 nm reaches a maximum upon addition of 6.0 equivalents of Cu^{2+} with an extinction coefficient (ε) of $2\times10^4\ M^{-1}cm^{-1}$ and then decreases with further addition of Cu^{2+}. The absorbance in the NIR region reaches a maximum ($\varepsilon = 5.2\times10^4\ M^{-1}cm^{-1}$) upon addition of a total of 8.0 equivalents of Cu^{2+} with a gradual blue shift of λ_{max} from 930 to 825 nm and that remains unchanged upon further addition of Cu^{2+}. The final solution color of ligand **1** is aqua colored. The complete colorimetric titration of ligand **1** with sequential addition of Cu^{2+} is shown in Figure 3.

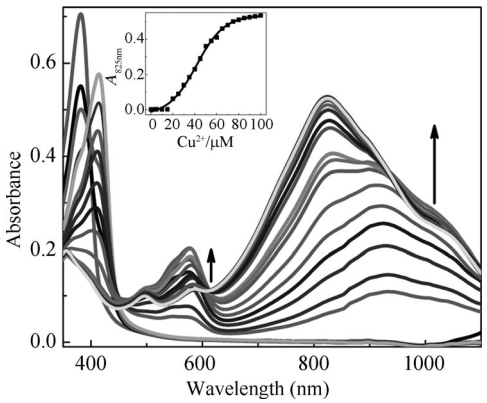


Figure 3. UV/Vis absorption spectra of julolidine–carbonohydrazone **1** (10.0 μM) on addition of different concentrations of Cu^{2+} (0, 2.5, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, and 100 μM) in aqueous medium (50 mM HEPES/CH_3CN, 6:4, v/v; pH 7.2). Inset: Absorbance at 825 nm as a function of [Cu^{2+}].

Cu^{2+} can be detected by an NIR response at least down to 20 μM when 10 μM of **1** is employed in an aqueous medium (50 mM, HEPES/CH_3CN, 6:4, v/v; pH 7.2). An absorbance peak in the NIR region would be useful for sensing Cu^{2+} in systems that contains unwanted interference of endogenous chromophores in the visible region. Ligand **2** (10 μM) exhibited an absorption band in the visible region (λ_{max} = 570 nm) accompanied by a well-distinguished NIR absorption band around 820 nm as shown in Figure 2. During sequential titration, two absorption bands appeared at 570 and 980 nm and correspond to the visible and NIR regions, respectively, after addition of 1.0 equivalent of Cu^{2+} (Figure 4). At this concentration of added Cu^{2+} the color of the solution of

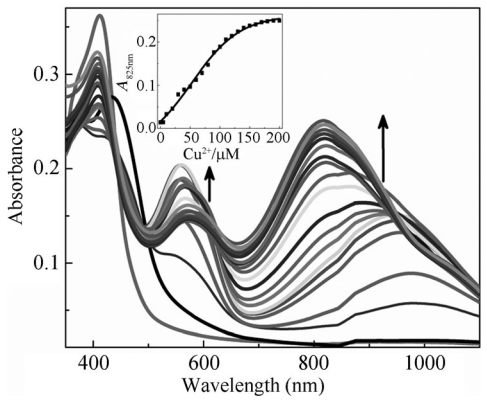


Figure 4. UV/Vis absorption spectra of julolidine–thiocarbonohydrazone **2** (10.0 μM) on addition of different concentrations of Cu^{2+} (0, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, and 200 μM) in aqueous medium (50 mM HEPES/CH_3CN, 6:4, v/v; pH 7.2). Inset: Absorbance at 825 nm as a function of [Cu^{2+}].

ligand **2** changes from greenish to light purple. The absorbance at 570 and 980 nm is enhanced by increasing the concentration of added Cu^{2+} from 1.5 to 2.0 equivalents and the purple-colored solution turns to light violet. The absorbance at 570 nm reaches the maximum at 5.0 equivalents of Cu^{2+} with an extinction coefficient (ε) of $2\times10^4\,M^{-1}cm^{-1}$ and a consequent increase in the intensity of the NIR absorption band was observed. At this composition of ligand **2** and Cu^{2+} the solution color changes from light violet to blue. Further addition of Cu^{2+} resulted in a gradual decrease in the absorbance at 570 nm as shown in Figure 4, whereas the absorbance in the NIR region increases and the band was gradually blue shifted to 820 nm and reaches a maximum with $\varepsilon=2.5\times10^4\,M^{-1}cm^{-1}$ upon overall addition of 15.0 equivalents of Cu^{2+} (Figure 4). The final solution color of ligand **2** becomes greenish aqua. By employing 10 μM concentration of ligand **2**, Cu^{2+} can be detected by an NIR response at least down to 10 μM in an aqueous medium. Apart from selective detection of Cu^{2+} using ligands **1** and **2**, different concentrations of Cu^{2+} can also be detected (Figure 5). The specific micromolar concentrations of added

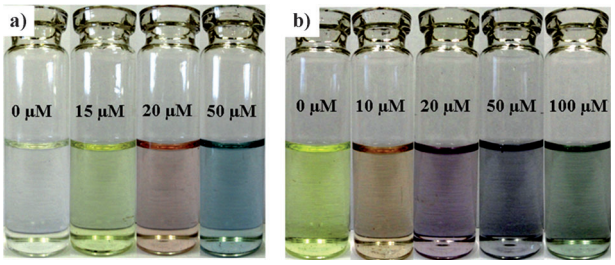


Figure 5. Detectable colorimetric change with increasing concentration of Cu^{2+} to the solution of a) julolidine–carbonohydrazone **1** (10.0 μM) and b) julolidine–thiocarbonohydrazone **2** (10.0 μM) in aqueous medium (50 mM HEPES/CH_3CN, 6:4, v/v; pH 7.2). The values indicated on the vials correspond to [Cu^{2+}].

Cu^{2+} gave distinct coloration to aqueous solutions of ligands **1** and **2** (Figure 5a and 5b, respectively). These colorimetric changes correspond to well-distinguishable visible absorbance spectra with a characteristic NIR signature in Figure 3 and Figure 4.

Competitive binding experiment: The selectivity of ligands **1** and **2** as Vis–NIR chemosensors for Cu^{2+} was studied in the presence of various competing metal ions. For this purpose ligands **1** and **2** were treated with a mixture of 5.0 equivalents of Cu^{2+} and 10.0 equivalents of all other metal ions. Data shown in Figure S2 (see the Supporting Information) confirms that there is no interference for the detection of Cu^{2+} in the presence of all other metal ions tested. The aqua color of the solutions of ligands **1** and **2** persist even in the presence of other metal ions in excess. Thus, ligands **1** and **2** can serve as highly selective colorimetric, as well as NIR, sensors for Cu^{2+} in the presence of most competing metal ions.

Stoichiometry of binding: The stoichiometry of binding of Cu^{2+} to ligands **1** and **2** were studied by various analytical techniques. Job's plots from UV/Vis absorbance data show 1:2 stoichiometric complexation between ligands **1** and **2** with Cu^{2+} (Figures S3 and S4 in the Supporting Information). This data was further supported by mass spectrometry analysis. The addition of Cu^{2+} to ligands **1** and **2** resulted in deprotonation of the hydroxyl functional groups of the julolidine moieties followed by complex formation. MALDI/TOF-MS shows the formation of a complex between deprotonated ligands and two copper ions: [$C_{27}H_{32}Cu_2N_6O_3+Na^+-2H^+$]: m/z: 635.17; calcd for $C_{27}H_{32}Cu_2N_6O_3+Na^+$: 637.1 and [$C_{27}H_{32}Cu_2N_6O_2S+Na^+-3H^+$]: m/z: 650.07; calcd for $C_{27}H_{31}Cu_2N_6O_2S+Na^+$, 653.07 (Figures S13 and S14 in the Supporting Information).

Response parameter and binding constant: The response parameter (α) which is defined as the ratio of free ligand concentration to the initial concentration of ligand is plotted as a function of Cu^{2+} concentration (see Figures S5 and S6 in the Supporting Information). This plot can serve as the calibration curve for the detection of Cu^{2+}. The association constant ($\log K_a$) of ligands **1** and **2** with Cu^{2+} was calculated as 13.52 and 14.36 M^{-1}, respectively, as determined from Li's equations.

pH dependence study: The influence of pH on the absorbance of ligands **1** and **2** upon complexation with Cu^{2+} was studied in aqueous medium (50 mM HEPES/CH_3CN, 6:4, v/v; Figures S7 and S8 in the Supporting Information).[27] Cu^{2+} can be clearly detected from the visible and NIR absorbance measurements by using ligands **1** and **2** over a pH range of 2–11. Therefore ligands **1** and **2** can be used for the environmental monitoring and biological detection of copper in most of commonly encountered pH ranges.

Fluorometric detection of Cu^{2+} using Julolidine–thiocarbonohydrazone 2: Fluorometric behavior of both the ligands **1** and **2** was studied upon addition of 20.0 equivalents of Li^+, Na^+, Ba^{2+}, Sr^{2+}, Mg^{2+}, Al^{3+}, Ca^{2+}, Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Ag^+, Cd^{2+}, Hg^{2+}, Pb^{2+}, and Cu^{2+} in aqueous medium (50 mM HEPES/CH_3CN, 6:4, v/v; pH 7.2). Ligand **1** did not show any specific changes in the fluorescence emission in the presence of Cu^{2+} and other metal ions used upon excitation at 402 nm (see Figure S9 in the Supporting Information). On the other hand, ligand **2** shows strong fluorescence emission around 535 nm upon excitation at 430 nm. The fluorescence intensity around 535 nm was quenched in the presence of only Cu^{2+} with 430 nm excitation, whereas no significant changes were observed in the fluorescence emission of ligand **2** in the presence of other metal ions under similar conditions (Figure 6a). The quenched fluorescence of ligand **2** was restored upon treating the ligand **2**–$2Cu^{2+}$ complex with ethylenediaminetetraacetic acid (EDTA; Inset: Figure 6a). Fluorescence emission of ligand **2** with sequential addition of increasing concentrations (0 to 50 μM)

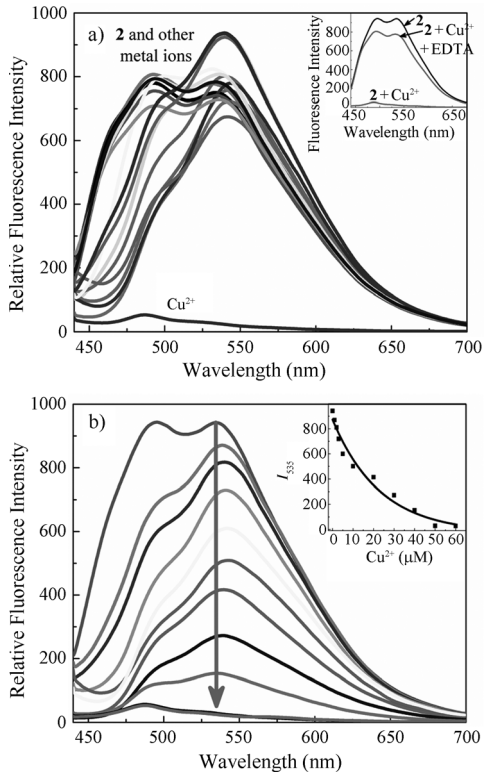


Figure 6. a) Fluorescence spectra of julolidine–thiocarbonohydrazone **2** (10.0 μM) and on addition of salts (20.0 equiv) of Li^+, Na^+, Ba^{2+}, Sr^{2+}, Mg^{2+}, Al^{3+}, Ca^{2+}, Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Ag^+, Cd^{2+}, Hg^{2+}, Pb^{2+}, and Cu^{2+} in aqueous medium (50 mM HEPES/CH_3CN, 6:4, v/v; pH 7.2). Inset: Fluorescence spectra of ligand **2** (10.0 μM), [**2**+Cu^{2+}] and [**2**+Cu^{2+}+EDTA] (10.0 μM). b) Fluorescence spectra of julolidine–thiocarbonohydrazone **2** (10.0 μM) on addition of different concentrations of Cu^{2+} (0, 1, 2, 3, 5, 10, 20, 30, 40, 50, and 60 μM) in aqueous medium. Inset: Intensity at 535 nm as a function of [Cu^{2+}].

of Cu^{2+} is shown in Figure 6b. This data shows that ligand **2** can easily detect copper ions at least down to 1.0 μM in aqueous medium. The reason behind the fluorescence quenching of **2** upon complexation with Cu^{2+} was understood by theoretical calculations as discussed in the Theoretical Study section. Ligand **2** can be used for detection of Cu^{2+} by fluorescence "on–off" phenomena in a wide pH (2–11) range in aqueous medium (see Figure S10 in the Supporting Information).

Theoretical Study

To understand the molecular mechanism underlying the experimentally observed photophysical characteristics of ligands **1** and **2** upon complexation with Cu^{2+}, we have investigated the structural, electronic, and optical properties using ab initio density functional theory (DFT) combined with time-dependent density functional theory (TDDFT) calculations as implemented in the Gaussian 03 package.[28] We have adopted the hybrid B3LYP[29] exchange and correlation functional using an effective core potential with the LANL2DZ[30] basis set for transition-metal Cu and the 6-31 g(d,p) basis set for all the other elements in the calculations. Job's plots and mass spectrometry analysis showed that ligands **1** and **2** form complexes with Cu^{2+} in a 1:2 stoichiometric ratio. Consequently, we have considered two magnetic Cu^{2+} ($S_z^t=1/2$) complexes with **1** and **2** for the DFT calculations. To find out the minimum energy magnetic ground state, unrestricted DFT calculations were performed considering both the high-spin ($S_z^t=1$) and low-spin ($S_z^t=0$) states within the broken symmetry (BS) approach.[31] We have also conducted vibrational energy calculations to confirm the local energy minimum structures. All of the DFT optimized geometries are shown in Figure 7.

Our calculated results show that the two magnetic Cu^{2+} centers are coupled with ferromagnetic (FM) spin alignments when forming complexes with both the ligands **1** and **2**. The FM state is stabilized over the antiferromagnetic (AFM) state by 5.61 kcal mol^{-1} for the ligand **1**+2Cu^{2+} complex and 3.58 kcal mol^{-1} for the ligand **2**+2Cu^{2+} complex (see Table S1 in the Supporting Information). To focus on the strength of the magnetic coupling constant (J), we make use of the simple 1D Heisenberg Hamiltonian for the interaction of two spins[32] and have obtained the FM coupling constants for both the complexes using the energy of low-spin states calculated within a BS approach (see

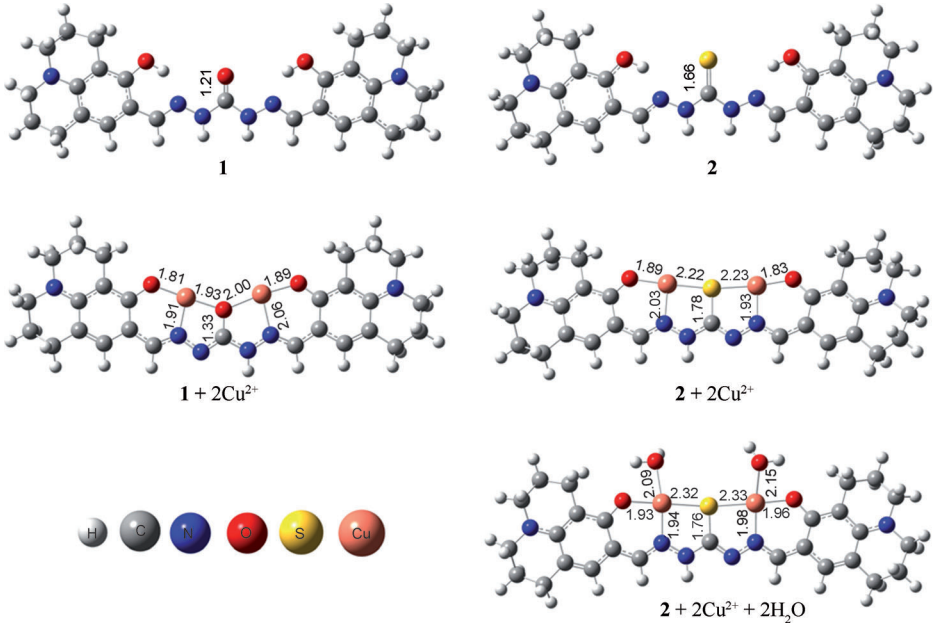


Figure 7. The optimized structures of julolidine–carbonohydrazone **1**, julolidine–thiocarbonohydrazone **2** and their Cu^{2+} complexes. The numbers in each structure show selected important bond-length parameters in Å.

Table S1 in the Supporting Information). As can be seen from Table S1 (in the Supporting Information), the relatively higher J value of **1**+2Cu^{2+} complex results from the strong super exchange interaction between the two magnetic Cu^{2+} ions mediated by the oxygen bridge as opposed to the sulfur bridge in **2**+2Cu^{2+} complex. The spin density distribution (See Figure S11 in the Supporting Information) for both complexes also confirms the FM spin alignments of the two copper ions. It should be noted that a significant amount of spin density is distributed over the delocalized conjugated π orbital in both complexes. To obtain an insight on the charge-density profile and bonding aspects, we have performed calculations of the natural bond orbital (NBO) and natural electronic configuration. A significant amount of charge transfer from ligand **1** and **2** to the Cu^{2+} center was observed (see Table S1 in the Supporting Information). This excess transferred electronic charge was found to be mainly localized on the d orbital of the two Cu^{2+} centers.

The experimentally observed peaks in the visible and NIR regions of the absorption spectra upon complexation with Cu^{2+} were studied by using TDDFT computations on ground-state optimized geometries of free ligands **1** and **2**, as well as their Cu^{2+} complexes. As shown in Figure 8, the TDDFT-computed excitation energies for free ligands (**1** and **2**) are in quantitative agreements with the experimental transition energies (Table S1 in the Supporting Information). From an analysis of frontier molecular orbitals (FMO) the lowest energy transition found consists of mainly promoting the electron from the delocalized highest occupied molecular orbital (HOMO) to the lowest unoccupied molecular orbital (LUMO) delocalized over the entire molecule (see Figure S12 in the Supporting Information). Interestingly, the observed shift in absorption peak position towards near-IR in forming a charge-transfer complex between Cu^{2+} and ligands **1** and **2** corroborates qualitatively with the experimental findings (Figure 8). The relevant FMOs responsible for these transitions are shown in Figure S12 (in the Supporting Information). The β-spin orbital contributes mainly to the observed new transition of these Cu^{2+} complexes. Here, it should be pointed out that the observed red shifting in transition energy upon Cu^{2+} chelating compared only qualitatively with the experimental results. This may be due to the fact that we have considered the complexation of two Cu^{2+} ions with each ligand (**1** and **2**) neglecting any coordination from water molecules. To compare and contrast the effect of water coordination on photophysical properties, we consider two water molecules coordinating with Cu^{2+} in the ligand **2**–2Cu^{2+} complex (Figure 7).[33] Interestingly, the shifting of the UV/Vis spectral peak position is less in comparison to the water-free coordination complex (Figure 8, bottom panel), which results in good agreement with the experimentally observed shift in peak position.

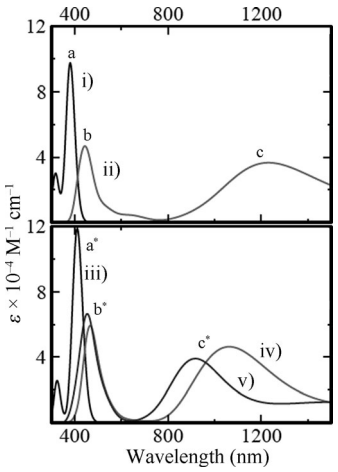


Figure 8. The calculated absorption spectra for **1** (i), **2** (iii) and their Cu^{2+} complexes: ii) **1**+2Cu^{2+}, iv) **2**+2Cu^{2+}, and v) **2**+2Cu^{2+}+2H_2O. The indices a (H→L), b (H-9(β)→L(β)), and c (H(β)→L(β)) and a*(H→L), b*(H(α)→L(α)), and c* (H(β)→L+1(β)) indicate contributions from major molecular orbital transitions corresponding to the peak as shown in Figure S12 (in the Supporting Information).

To investigate the mechanism (energy transfer and/or charge transfer process) of fluorescence-quenching behavior upon chelation with Cu^{2+}, we have performed excited-state geometry optimization and subsequent single-point transition-energy calculations employing the TDDFT method as implemented in the Gaussian suite of programs for compound **2** in the presence of Cu^{2+} and two explicit water molecules. From an analysis of FMOs corresponding to the lowest energy transition the fluorescence quenching by Cu^{2+} could be rationalized in terms of the occupancy of FMOs. As shown in Figure 9, the HOMO (β)→LUMO+**1** (β), HOMO−**1** (β)→LUMO+**1** (β), and HOMO−**2** (β)→LUMO+**1** (β) electronic excitations are found to be relevant for the lowest energy fluorescence process showing predominantly ligand-to-metal charge transfer (LMCT) and their contributions to the lowest energy excitation are 24, 18, and 9%, respectively. The transferred charge is mainly localized on the two Cu^{2+} centers, as well as on its nearby atoms. Note that the calculated values are comparatively small and strongly dependent on the method and basis sets used for their computations. Depending on this, the extent of excited-state charge transfer may significantly alter and cause the fluorescence quenching. These excitations correspond to the charge transfer from the excited state of ligand

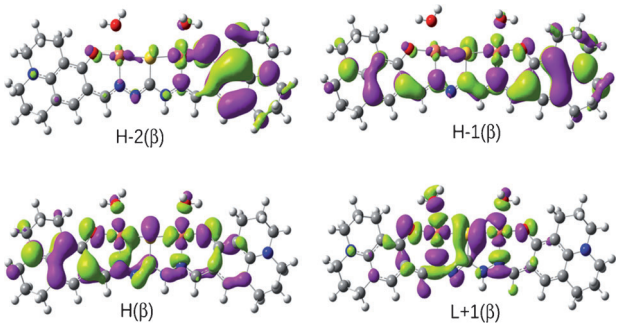


Figure 9. The relevant frontier molecular orbitals (FMOs) of the **2**+2Cu^{2+}+2H_2O complex corresponding to the excited-state charge-transfer process. The symbols H and L represent the highest occupied molecular orbital and lowest unoccupied molecular orbital, respectively.

2 to the Cu^{2+} center (LMCT) and thus provide a pathway for the nonradiative deactivation of the excited state. Thus, fluorescence-quenching behavior can be accounted for primarily due to the excited-state ligand (**2**) to metal (Cu^{2+}) charge transfer (LMCT) processes.

Bioimaging

Subsequent experiments proved the ability of julolidine–thiocarbonohydrazone (**2**) to track Cu^{2+} levels in living cells by using fluorescence microscopy. HEK293T cells were grown to 50% confluency in a 30 mm dish with Dulbecco's modified eagle medium (DMEM) and 10% fetal bovine serum (FBS) at 37°C and 5% CO_2. The cells were washed thrice with phosphate-buffered saline (PBS) and stained with ligand **2** (10 μM) in the growth media without FBS for 10 min. The adherent cells were washed thrice with PBS to remove excess stain. Fluorescence emission was observed in the optical window at 450–650 nm as shown in Figure 10. The stained cells were subsequently supplemented with $Cu(ClO_4)_2{\cdot}6H_2O$ (10 μM) in DMEM without FBS at 37°C and 5% CO_2 for 10 min and then intracellular fluorescence was almost completely suppressed. Finally, the excess $Cu(ClO_4)_2{\cdot}6H_2O$ was washed off with PBS and then supplemented with EDTA (10 μM) in DMEM without FBS for 10 min at 37°C to recover the fluorescence. Since the cells stained with ligand **2** show a clear-cut cytoplasmic localization these studies clearly suggested that ligand **2** is cell-permeable and can respond to copper ions within living cells.

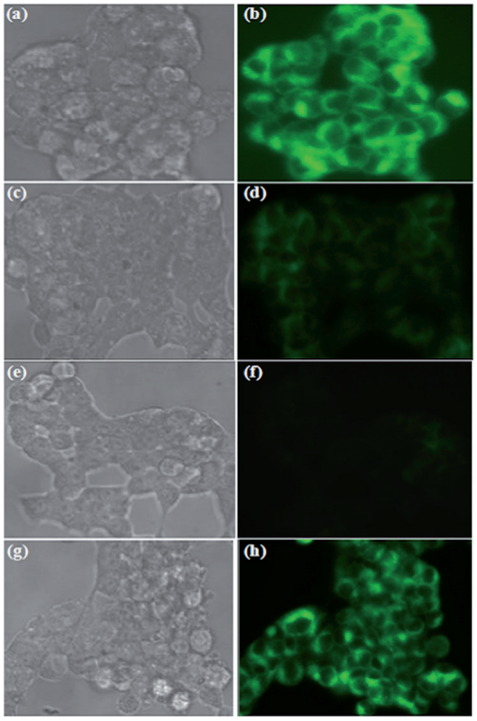


Figure 10. Fluorescence imaging of Cu^{2+} in live HEK293T cells. a) Bright-field transmission image and b) fluorescence image of HEK293T cells incubated with julolidine–thiocarbonohydrazone **2** (10 μM) for 5 min. c) and e) Bright-field transmission images and d) and f) fluorescence-quenched images of HEK293T cells treated with julolidine–thiocarbonohydrazone **2** and incubated with $Cu(ClO_4)_2{\cdot}6H_2O$ (10 μM) for 5 and 10 min, respectively. g) Bright-field transmission image and h) restoration of the fluorescence of ligand **2** with the addition of EDTA (10 μM).

Conclusion

We have established a design strategy to develop new Vis–NIR chemosensors for Cu^{2+} detection. Julolidine–carbonohydrazone **1** and julolidine–thiocarbonohydrazone **2** can independently detect Cu^{2+} with high selectivity in the presence of other metal ions with a characteristic NIR signature in aqueous medium. Ligands **1** and **2** can be used for detection with the naked eye of different concentrations of Cu^{2+}, since they exhibit distinct colors in aqueous solution and therefore one can easily assess the rough concentration levels of Cu^{2+} in analyte samples. In addition, fluorescent julolidine–thiocarbonohydrazone **2** selectively senses Cu^{2+} by fluorescence quenching in the presence of all other metal ions under aqueous conditions. The quenched fluorescence emission of ligand **2** can be restored by the addition of EDTA. We have further demonstrated the utility of ligand **2** as a reversible biosensor by employing it for living-cell imaging of Cu^{2+} in HEK293T cells. Results obtained from theoretical calculations corroborate the experimental findings and provide us with a detailed microscopic understanding of the observed NIR and fluorescence properties of ligands **1** and **2** upon Cu^{2+} chelation.

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