Highly fluorescent probe for copper (II) ion based on commercially available compounds and live cell imaging

Duraisamy Udhayakumari a, Sivan Velmathi a,*, Yi-Ming Sung b, Shu-Pao Wu b

a Organic and Polymer Synthesis Laboratory, Department of Chemistry, National Institute of Technology, Tiruchirappalli 620 015, India
b Department of Applied Chemistry, National Chiao Tung University, Hsinchu, Taiwan 300, Republic of China

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The commercially available 2, 3-diaminophenazine, 1, 2-diamino-anthraquinone and 2, 4-dinitrophenylhydrazine (S1–S3) act as a colorimetric, fluorescent probes for selective detection of Cu(II) ions in aqueous medium over other metal ions. In presence of Cu(II), S1 shows fluorescent turn-off by the paramagnetic effect. S2 and S3 with Cu(II) ions show fluorescent turn-on due to the inhibition of photoinduced electron transfer mechanism. The fluorescent probes S1–S3 detect Cu(II) ions in aqueous solution at nanomolar levels. Theoretical calculations were employed to understand the sensing mechanism of the sensors towards Cu(II). S1, S2 and S3 were further applied for biological imaging to confirm that it can be used as a fluorescent probe for monitoring Cu(II) in living cells, and demonstrated its value in practical applications such as environmental and biological systems.

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1. Introduction

The detection of metal ions is very important for analytical, environmental and biomedical applications due to their deleterious effects on human health and ecosystems [1–3]. Ion exchange chromatography, biochemical, electrochemical techniques and spectroscopic methods are used for the detection of metal ions, but those methods are quite difficult, higher in cost and unsuitable for on-line or field monitoring. Fluorescent sensors have more advantage such as simplicity, high sensitivity and selectivity. Therefore the fluorescence sensing of simple molecules has gained much attention in the field of environmental, medicine and biological chemistry [4–7]. Some of the metals play important role in living system, electrical and electronic industry. Copper plays a critical role as a catalytic cofactor for a variety of metalloenzymes, including superoxide dismutase, cytochrome c oxidase, tyrosinase and nuclease. Copper is one of the toxic metals [8–10], irregular ingestion of copper could cause lethargy and increased blood pressure [11–15]. The high level of copper causes neurodegenerative diseases such as Alzheimer’s, Parkinson’s and is also suspected to cause amyloid precipitation and toxicity [16–23]. Various analytical

methods and number of fluorescent probes have been developed for the detection of copper ions selectively as well as sensitively. But, only a few chemosensors in which the binding of a Cu(II) ion causes an increase in the fluorescence, have been reported [24–28]. However, the development of fluorescent sensor for copper ions in aqueous medium is an important task. In recent years, the design and syntheses of selective colorimetric and fluorescent copper probes have received great attention. The above mentioned probes are prepared by using certain organic synthesis process (single or multi step). But commercially available compounds have the advantages of cost effectiveness, simplicity, no need of organic synthesis process and field work applicability. Recently Lijun Qu et al., have reported a commercially available 1, 8-diamino naphthalene based fluorescence chemosensor for copper ions [29].

Herein, we report commercially available aromatic amines 1, 2-diaminophenazine, 1, 2-diamino-anthraquinone and 2, 4-dinitrophenylhydrazine (S1–S3) which detect copper ions selectively under four different channels (colorimetric, absorption, emission and bioimaging) in presence of other metal ions. Using these commercial available compounds as a starting material, many organic compounds are synthesized and reported mainly for anion sensing [30–35]. First time we report the commercially available 1, 2-diaminophenazine, 1, 2-diamino-anthraquinone and 2, 4-dinitrophenylhydrazine (S1–S3) as selective copper ion sensors. The fluorescent probes S1–S3 detect Cu(II) ions in aqueous solution even at nanomolar levels. As the detection limit of S2 and S3 is

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0.15 × 10⁻⁹ M L⁻¹. These probes are highly sensitive. In case of S1, the quenching of the fluorescence emission is observed due to the paramagnetic nature of Cu²⁺ ions. But, enhancement of the fluorescence emission was observed in case of S2 and S3 bound with Cu²⁺.

2. Materials and spectroscopic methods

2, 3-diaminophenazine, 1, 2-diamino-anthraquinone and 2, 4-dinitrophenylhydrazine, iron (III) chloride, cobalt (II) chloride, nickel (II) chloride, copper (II) chloride, zinc (II) acetate, cadmium (II) acetate, lead (II) acetate, mercury (II) nitrate, manganese (II) acetate, tin (II) chloride and chromium (III) chloride were purchased from Sigma–Aldrich and used as such. 5 × 10⁻⁵ M solution of S1-S3 were prepared in acetonitrile (CH₃CN). 1.5 × 10⁻⁵ M solution of the cations was prepared in H₂O. To 3 ml of S1 taken in the UV cuvette, 0.2 eq. (20 µL) − 2 eq. (200 µL) of Cu²⁺ solution was added. The same solutions were used for fluorescent titrations. ¹H and ¹³C NMR were obtained on a Bruker 400 MHz spectrometer using CDCl₃ as solvent and tetramethylsilane (TMS) as an internal standard, IR spectrum was recorded by KBr pellet method on a Perkin–Elmer Spectrum One FTIR spectrometer. UV–visible spectra were recorded in UV–2600 a UV–vis spectrophotometer using 1 cm path length quartz cell. Fluorescence emission spectra were recorded in a Shimadzu RF-5301 PC spectrofluorophotometer at a scan rate of 500 nm/min. Excitation wavelength chosen was 300 nm for S1 and S3 and 400 nm for S2. Fluorescence imaging was performed with a Leica TCS-SP5-X A0BS Confocal microscope.

3. Results and discussion

3.1. Colorimetric sensing studies

Though the sensors S1-S3 (Fig. 1) are well known and commercially available compounds, ¹H NMR spectroscopic studies (see supporting information) were done to check for the purity of the compounds. The selective detection of environmentally active metal ions is investigated by visual, optical, fluorescence spectroscopy and bioimaging method. The sensors S1-S3 were prepared in 5 × 10⁻⁵ M concentration in CH₃CN and all cations were prepared in 1.5 × 10⁻⁵ M concentration in H₂O. Sensors S1-S3 were treated with various metal ions like Fe³⁺, Co²⁺, Ni²⁺, Cu²⁺, Zn²⁺, Cd²⁺, Pb²⁺, Hg²⁺, Mn²⁺, Sn²⁺ and Cr³⁺ to study the sensitivity and selectivity towards particular metal ions over other metal ions. For the addition of 100 µL of all metal cations into S1-S3, the presence of Cu²⁺ ions shows colorimetric turn-off response from green to colorless for S1 (Fig. 2a) and S3 (Fig. 2c) and pink to colorless for S2.

![Fig. 1. Structure of sensors 1, 2 and 3.](image1)

![Fig. 2. a. Color changes of sensor 1 (5 × 10⁻⁵ M solution in CH₃CN) before and after the addition of 100 µL of respective cations (1.5 × 10⁻³ M solution in H₂O). b. Color changes of sensor 2 (5 × 10⁻⁵ M solution in CH₃CN) before and after the addition of 100 µL of respective cations (1.5 × 10⁻³ M solution in H₂O). c. Color changes of sensor 3 (5 × 10⁻⁵ M solution in CH₃CN) before and after the addition of 100 µL of respective cations (1.5 × 10⁻³ M solution in H₂O) (From the left to right: R, R + Fe³⁺, R + Co²⁺, R + Ni²⁺, R + Cu²⁺, R + Zn²⁺, R + Cd²⁺, R + Pb²⁺, R + Hg²⁺, R + Mn²⁺, R + Sn²⁺ and R + Cr³⁺).](image2)
ions, calculated plots Fig. 3.3. Performance significant which Cu2+ ions. Two equivalents of all the metal ions were added to the Cu2+ ion complex of S1–S3 solution, but no changes were observed in the color of the S1–Cu2+, S2–Cu2+ and S3–Cu2+ complex. This result is an added evidence for the high stability of the Cu2+ ion sensing, even in presence of other cations without any interference (see supporting information).

3.2. UV–vis spectroscopic studies

The binding ability of sensors (S1–S3) was further studied by optical spectroscopy. The UV–vis spectra of S1, S2 and S3 show strong absorption bands at 400 nm, 500 nm and 375 nm respectively. Fig. 3a–c shows the absorption spectrum of S1–S3 with metal ions in aqueous medium. With the addition of Cu2+ into sensors (S1–S3), the intensities of the bands 400 nm (S1), 500 nm (S2) and 375 nm (S3) have been reduced. The other metal ions like Fe3+, Co2+, Ni2+, Zn2+, Cd2+, Pb2+, Hg2+, Mn2+, Sn2+ and Cr3+ ions did not show any optical changes with sensors S1–S3. From this, it is clear that S1–S3 can detect Cu2+ selectively in presence of other metal ions. The incremental addition of Cu2+ ions into sensors (S1–S3), the corresponding absorption bands are gradually decreased (see supporting information) with isosbestic point at 425 nm for S2, 315 nm for S3. The relative absorbance of sensors (S1–S3) with all metal ions shows the selectivity of Cu2+ ions over other metal ions (see supporting information). Interestingly, the solution turned to colorless. The interaction and interference of Cu2+ ions with S1–S3 were studied. Cu2+ was selectively in presence of the other metal ions. We have titrated S1–S3 with 2 eq Cu2+ in presence of other metal ions like Fe3+, Co2+, Ni2+, Zn2+, Cd2+, Pb2+, Hg2+, Mn2+, Sn2+ and Cr3+. The excitation wavelength selected for S1 and S3 is 300 nm and for S2 is 400 nm. As shown in Fig. 4a, a clear fluorescence quenching is observed upon the addition of 100 μL of Cu2+ ions into S1. But the interaction of other mentioned ions with S1 showed very little fluorescence intensity changes. The intensity of fluorescence band at 500 nm for S1 (Φ = 0.51) decreased gradually during the incremental addition of Cu2+ ions (Φ = 0.0033) (see supporting information). Fluorescence quenching is measured quantitatively with Stern–Volmer equation. The calculated Stern–Volmer constant Ksv (M−1) of sensor 1 with Cu2+ is 8.0 × 107 and linear regression correlation coefficient is 0.963, which suggests a linear correlation. The linearity in Stern–Volmer plots are interpreted in terms of the sphere of action defined in the dynamic quenching model. The fluorescence of S2 and S3 is highly enhanced in the presence of Cu2+. Fig. 4b and c show the distinct fluorescence enhancement for S2–S3 in presence of 100 μL Cu2+ ions. But the presence of other metal ions induces no significant change in the emission spectrum. The insert figures of Fig. 4a–c show the color change photographs for Cu2+ and the other metal ions under illumination with a 365 nm UV lamp. The gradual addition of Cu2+ ions into S2 (Φ = 0.035), the fluorescence band at 440 nm shifted (red Shift) to 475 nm with high intensity (Φ = 0.9) (see supporting information) and a clear isosbestic point at 580 nm. The result revealed the coordination of Cu2+ ions with the amine (S2). A fluorescence titration experiment of S3 (Φ = 0.15) with Cu2+ ions was carried out. A linear increase in the fluorescence
intensity was observed with the increasing concentration of Cu$^{2+}$ ions ($\phi = 0.8$). Fig. 4d–f shows the relative fluorescence of S1–S3 with all metal ions. The colorimetric responses of the S1–S3 to other copper salts like Cu (AcO)$_2$, CuCl$_2$, Cu(SO$_4$)$_2$, Cu(ClO$_4$)$_2$ and Cu(NO$_3$)$_2$ was carried out (see supporting information). There is no divergence in fluorescence intensity in the presence of other copper salts. From this result it can be inferred that Cu$^{2+}$ ions selectively sense in the presence of counter anions (see supporting information).

3.4. Binding constant, detection limit and jobs plot studies

Benesi–Hildebrand method has become one of the most common strategies for determining association constants based on the absorbance spectra. Using Benesie Hildebrand expression, the binding constants of S1–S3 with Cu$^{2+}$ were investigated. The corresponding measured absorbance $[1/(A - A_0)]$ for S1–S3 has varied as a function of $1/[\text{Cu}^{2+}]$ in a non-linear relationship. The binding constants of S1–S3 with Cu$^{2+}$ are $3.3 \times 10^3$, $3.3 \times 10^2$ and $2.8 \times 10^2$ respectively. Job’s plot studies indicate host–guest molecular complexation, the sensors S1 and S2 with Cu$^{2+}$ ions form 1:1 stoichiometry whereas S3 with Cu$^{2+}$ ions form 2:1 stoichiometry (Fig. 5) (see supporting information). Using fluorescence titration experiments of S1–S3 with Cu$^{2+}$ ions, the detection limits were calculated. The detection limit was then calculated with the equation: detection limit = 3$\sigma_{bi}$/m, where $\sigma_{bi}$ is the standard deviation of blank measurements and $m$ is the slope of the intensity versus sample concentration. The detection limits of Cu$^{2+}$ ion with S1–S3 are 0.23 sub-nM, 0.15 nM, 0.15 nM respectively. The stoichiometries of the complexes discussed above coincide with the previous reports [29,36]. The detection limit, interference with other metals of S1, S2 and S3 was compared with the previously reported probes (Table 1) [37–40].

3.5. Effect of pH

The influence of pH on S1–S3 was studied using fluorescence spectroscopic method (see supporting information). In S1 over a pH range of 4–8, the fluorescence quenching band at 520 nm was unchanged. When the pH value exceeded 8, the intensity at 520 nm increased. This was due to the dissociation of S1–Cu$^{2+}$ complexes. At pH values less than 4, absorbance was almost negligible; evidently the S1–Cu$^{2+}$ complexes do not exist over this pH range. For sensor 2–Cu$^{2+}$ complexes, the intensity at the wavelength 500 nm suddenly increased at pH 5.0 and reached maximum at pH 8.0. This indicates that the formation of S2–Cu$^{2+}$ complexes is a deprotonation process. When the pH value exceeded 8.0, the intensity
at 475 nm gradually decreased, due to the dissociation of S2–Cu$^{2+}$ complexes. The effect of pH on the formation of S3–Cu$^{2+}$ complexes was also studied. For the S3–Cu$^{2+}$ complexes, the emission at the wavelength 380 nm increased sharply at pH 5.0 and no intensity changes up to pH 9.5. This also indicates that the formation of S3–Cu$^{2+}$ complexes is a deprotonation process.

3.6. Sensing mechanism

The coordination of metal ions could cause an enhancement of the fluorescence or quenching of the fluorescence intensity. The enhancement of fluorescence emission is called Chelation Enhanced Fluorescence effect (CHEF). The quenching of the fluorescence is called Chelation Enhancement Quenching effect (CHEQ). Both effects can be coupled with a red or blue shift of the emission band. S1 with Cu$^{2+}$ shows the fluorescence quenching; via paramagnetic fluorescence quenching. In a wide variety of metal complexes, the forbidden intersystem crossing (ISC) becomes faster due to the presence of a d$^0$ Cu (II) ion paramagnetic atom in the proximity of the fluorophore. These complexes undergo inter system crossing by excitation, from S1 to T1 state of the fluorophore that can be deactivated by bimolecular non-radiative processes (Fig. 6). In case of S2 and S3, binding with Cu$^{2+}$ ions, fluorescence enhancement occurred due to the Photoinduced Electron Transfer (PET) mechanism. S2 and S3 show weak fluorescence, PET takes place from the lone pair (N) of the coordinating atoms to the HOMO of the excited fluorophore. The presence of a Cu$^{2+}$ ion lowers the energy of the lone pair involved in the coordination preventing the PET, thus causing the switch-ON mechanism of the fluorescence (Fig. 7).

3.7. Theoretical calculation study

Density Functional Theory (DFT) calculations have been used to understand the behaviour of S1–S3 with the Cu$^{2+}$ ions. Highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO) for S1–S3 and their metal complexes have been generated from optimized structures of the sensors S1–S3 and their respective metal complexes (Fig. 8). The structural optimization and the computational calculations were carried out using Gaussian09 quantum chemistry package and results were viewed with GaussView5 GUI. The appropriate choice of model chemistry for the computation of electronic structure property is indispensable. In the present scenario, Density Functional Theory (DFT) supported B3LYP/6-31G (d, p) model chemistry was implemented particularly for Schiff base ligands under gas phase condition. But its metal complexes were modeled using effective core potential (ECP) based LANL2DZ (Los Alamos National Laboratory 2-double-z–Acronyms)
basis set to optimize the geometry of the complex. The metal atom present in the complex was treated under LANL2DZ where as other atoms are manipulated with B3LYP/6-31G (d, p) level of theory. DFT calculations were carried out to investigate the change of the absorption spectra upon the addition of Cu$^{2+}$. From the energy level diagram (Fig. 9), it can be seen that the addition of the metal ions lead to the stabilization of the HOMO and LUMO of the sensor molecule. From the calculated results, the energy levels of HOMO

Table 2
Energies of HOMO and LUMO of the S1–S3 and complexes.

<table>
<thead>
<tr>
<th>Ligands &amp; metal complexes</th>
<th>HOMO (eV)</th>
<th>LUMO (eV)</th>
<th>$\Delta E$ (eV)</th>
<th>Theoretical wavelength (nm)</th>
<th>Observed wavelength (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>-5.484</td>
<td>-2.524</td>
<td>2.960</td>
<td>405 nm</td>
<td>414 nm</td>
</tr>
<tr>
<td>S1 + Cu(II)</td>
<td>-6.978</td>
<td>-2.828</td>
<td>4.149</td>
<td>292 nm</td>
<td>308 nm</td>
</tr>
<tr>
<td>S2</td>
<td>-5.046</td>
<td>-2.568</td>
<td>2.478</td>
<td>484 nm</td>
<td>499 nm</td>
</tr>
<tr>
<td>S2 + Cu(II)</td>
<td>-6.833</td>
<td>-3.691</td>
<td>3.142</td>
<td>382 nm</td>
<td>373 nm</td>
</tr>
<tr>
<td>S3</td>
<td>-6.391</td>
<td>-2.955</td>
<td>3.436</td>
<td>349 nm</td>
<td>352 nm</td>
</tr>
<tr>
<td>S3 + Cu(II)</td>
<td>-7.816</td>
<td>-3.863</td>
<td>3.952</td>
<td>343 nm</td>
<td>299 nm</td>
</tr>
</tbody>
</table>
and LUMO of S1–S3 increased after the addition of Cu^{2+} ions when compared to that of the free S1–S3 (Table 2). For Cu^{2+} complexes of S1–S3, the absorption peaks predicted by the theoretical methods is blue-shifted compared with those of the independent probes, which is in good agreement with the experimental observations.

3.8. Cell imaging study

To determine the applicability of S1–S3 for practical applications, bioimaging studies in living cells were done. HeLa cells were grown in H-DMEM (Dulbecco’s Modified Eagle’s Medium, high glucose) supplemented with 10% FBS (Fetal Bovine Serum) in an atmosphere of 5% CO_{2} at 37 °C. The cells cultured in DMEM were treated with 10 mM solutions of Cu^{2+} (2 μL; final concentration: 20 μM) dissolved in sterilized PBS (Phosphate Buffered Saline) (pH 7.4) and incubated at 37 °C for 30 min. The treated cells were washed with PBS (2 mL × 3) to remove remaining metal ions. DMEM (2 mL) was added to the cell culture, which was then treated with a 10 mM solution of chemosensor S1 (2 μL; final concentration: 20 μM) dissolved in DMSO. The samples were incubated at 37 °C for 30 min. The culture medium was removed, and the treated cells were washed with PBS (2 mL × 3) before observation. For S1,
the cells were excited with a white light laser at 405 nm, and emission was collected at 510 Å ± 25 nm for S1 and at 463 Å ± 25 nm for S2 and S3. From this study, the green fluorescence was observed from cells HeLa cells stained with S1 and copper ions (Fig. 10) whereas blue fluorescence was observed from HeLa cells stained with S2 (Fig. 11), S3 (Fig. 12) and copper ions. The significant green and blue fluorescence from the intracellular region proves that S1–S3 are applicable for imaging Cu²⁺ in living cells. The bioimaging in the HeLa cells confirm the fluorescence quenching and fluorescence enhancement with excellent cell permeability. It shows that S1–S3 are biocompatible in nature and can be used for detecting Cu²⁺ ions in cell rapidly.

4. Conclusion

In conclusion, we have brought out the potential of commercially available compounds (S1–S3), for the selective detection of Cu²⁺ ions over the other interfering metal ions. The sensors (S1–S3) showed specific selectivity for Cu²⁺ ions in aqueous medium. Investigation of the recognition mechanism indicates that S1 and S2 recognize Cu²⁺ by forming a stable 1:1 complex whereas S3 forms a stable 2:1 S3–Cu²⁺ complex. The detection limits of S1–S3 towards Cu²⁺ ions were 0.23 sub-nM (S1), 0.15 nM (S2) and 0.15 nM (S3) respectively, which indicate that the sensors S1–S3 are the potential colorimetric sensors for monitoring Cu²⁺ ions in physiological and environmental systems. The fluorescence properties of sensors in the presence of Cu²⁺ ions were evaluated. The significant fluorescence quenching was probably caused by S1 coordinated with Cu²⁺, the paramagnetic effect from spin-orbit coupling of the Cu²⁺. On the other hand, the fluorescence of S2 and S3 was remarkably enhanced upon addition of Cu²⁺. This enhancement may be due to the inhibition of photoinduced electron transfer mechanism (PET). The value of these sensor systems (S1–S3) has been demonstrated by its use in detecting Cu²⁺ ions in living cells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.snb.2014.03.063.

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Cu(II) selective chemosensor, 


Biographies

Ms. Duraisamy Udhayakumari was born in 1989. She received her Under Graduate degree from Vellore Institute of Technology, Chennai in the year 2001. Currently, she is doing her PhD in the same institute under the guidance of Dr. S. Velmathi. Her research is focused on the synthesis of novel chromogenic and fluorogenic organic receptors for the recognition of anions/cations and their utilizations in the real samples.

Dr. Sivan Velmathi was born in 1974 in Chennai, India. She received her PhD degree in Organic Chemistry from the University of Madras in the year 2001. Currently, she is working as an Associate Professor in Department of Chemistry (Organic and Polymer Synthesis Laboratory), National Institute of Technology, Trichy. Her research focuses on the synthesis of new chromogenic and fluorogenic organic receptors for the recognition of anions/cations and their applications in real samples.

Dr. Shu-Pao Wu had Ph.D. in 2004, Department of Chemistry, The Ohio State University, USA. Currently, he is working as an Associate Professor in Department of Applied Chemistry National Chiao Tung University, Taiwan, Republic of China. Current interests: metal ion chemosensors and AIB.