Retraction for *Analyst*:

Retracted article: Pyrene-hydrazone based chemosensors for Cu^{2+} and their application in cancer cell imaging

Sellamuthu Anbu, Sankarasekaran Shanmugaraju, Rajendran Ravishankaran, Anjali A. Karande and Partha Sarathi Mukherjee

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We the authors Sellamuthu Anbu, Sankarasekaran Shanmugaraju, Rajendran Ravishankaran, Anjali A. Karande and Partha Sarathi Mukherjee hereby wholly retract this *Analyst* article. This decision is due to the fact that recently it has been brought to our attention that the work reported has partial overlap with the ongoing research of another group. The overlap of results was not intentional and this *Analyst* article is being retracted by the authors in order to maintain the accuracy of the scientific record.

Signed Sellamuthu Anbu, Sankarasekaran Shanmugaraju, Rajendran Ravishankaran, Anjali A. Karande and Partha Sarathi Mukherjee, Indian Institute of Science, 20th December 2012.

This retraction is endorsed by May Copsey, Editor. Retraction published 3rd January 2013.

Analyst

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Pyrene-hydrazone based chemosensors for Cu^{2+} and their application in cancer cell imaging

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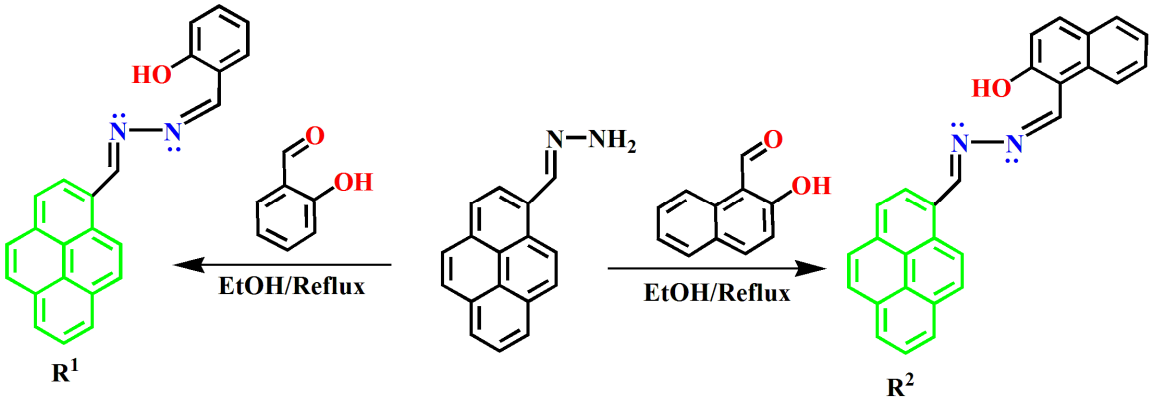
Abstract: Two pyrene-hydrazone based fluorescent chemosensors **R[1]** [pyrene-1-(2-hydroxy)phenylhydrazone] and **R[2]** [pyrene-1-(2-hydroxy)naphthylhydrazone] have been synthesized and examined as selective and sensitive receptors for Cu^{2+} ion in aqueous medium. Quantification of the fluorescence titration profile indicated that these newly synthesized receptors **R[1]** (58.5 ppb) and **R[2]** (1.26 ppb) can detect the presence of Cu^{2+} ion even at very low concentrations. In addition, the propensity of these receptors as bio-imaging fluorescent probes to detect Cu^{2+} ion in human cervical HeLa cancer cell lines has been demonstrated.

Introduction: Research on suitable chemosensor that can show highly selective and sensitive binding affinity towards transition metal ions has attracted wide interest due to their vital roles in various biological and environmental processes.[1-3] Of the different kinds of sensing, fluorescent based technique has many advantages due to high sensitivity, straightforward application and real-time monitoring with fast response time.[4] In light of these, substantial efforts have been focused and several chemosensors have been developed for sensing of particular metal ions both *in vitro* and *in vivo*.[5-6] In

particular, the development of sensors for the selective and very rapid detection of Cu^{2+} ion has made a special attention in recent past.[7] Copper is an essential trace element in the human body and is commonly found as Cu^{2+} in natural water.[7] Moreover, in living systems the brain needs much higher level of copper compared to other parts of the body under normal physiological conditions,[8-9] but at the same time copper deficiency may lead to a wide variety of neurological problems. Excess accumulation of copper causes various disorders associated with neurodegenerative ailments, Alzheimer's, Wilson's, and Menke's diseases.[10-15] Due to their important roles in several biological functions, the search for selective and sensitive fluorescent sensors for Cu^{2+} ions is an appealing field of research. In light of this, several pyrene based chemosensors[16-22] have been reported in the recent past. Although several sensors for Cu^{2+} are known,[23-26] slow response time, low sensitivity and lack of high selectivity including cytotoxicities of these sensors limit them to use for the real-time practical applications. Moreover, selective fluorescent chemosensors for Cu^{2+} with the detection limit of ppb level are relatively less known in the literature.[27] Therefore, the judicious design and synthesis of a selective fluorescence chemosensor for the rapid detection of Cu^{2+} ions in both biological and environmental systems is quite essential. Herein we report two pyrene hydrazone-based fluorogenic chemosensors $\mathbf{R^1}$ and $\mathbf{R^2}$ for Cu^{2+} ions with the detection limit of ppb level even in the presence of several other chalcophilic metal ions and its potential application in human cancer (HeLa) cells bio-imaging.

Results and discussion

Synthesis of fluorescent probes R^1 and R^2: The receptors R^1 and R^2 were synthesized in quantitative yields by condensation of pyrene-1-hydrazide with salicyladehyde and 2-hydroxy-1-naphthaldehyde in ethanol at 85 ºC, respectively (Scheme 1). The structures of R^1 and R^2 were characterized by FTIR, multinuclear (^1H, ^{13}C) NMR, HRMS and elemental analyses (Fig. S1–S7).



Scheme 1. Synthesis of the receptors R^1 and R^2.

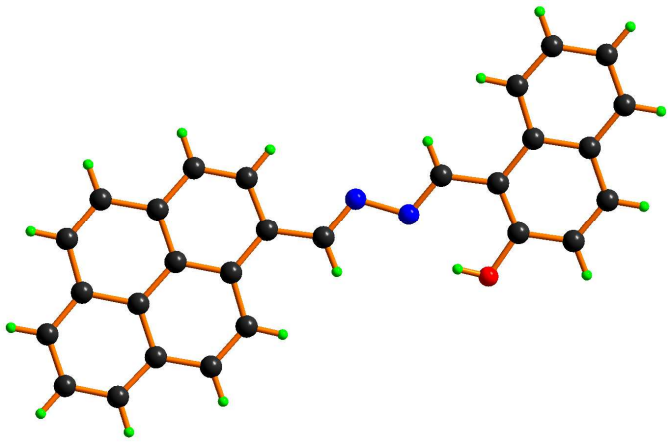


Fig. 1. X-ray crystal structure of receptor R^2 (color codes: C = dark grey, O = red, N = blue, H = green).

Finally, the molecular structure of the receptor $\mathbf{R^2}$ was confirmed by single crystal X-ray diffraction analysis. The diffraction quality single crystals were obtained by slow evaporation of a concentrated CH_3CN solution of $\mathbf{R^2}$ at room temperature. $\mathbf{R^2}$ was crystallized in the *P*21 space group with four formula units per asymmetric units. A ball and stick representation of the structure of $\mathbf{R^2}$ is depicted in Fig. 1.

Sensing studies

Sensing ability of the receptors $\mathbf{R^1}$ and $\mathbf{R^2}$ for different metal cations as their chloride salts was monitored using UV-vis absorption and fluorescence spectroscopy analysis. Since the receptors $\mathbf{R^1}$ and $\mathbf{R^2}$ are not highly soluble in water, all the titration studies were carried out in H_2O/CH_3CN (2:1, v/v) solvent mixture. The electronic absorption spectrum of $\mathbf{R^1}$ (5 μM) exhibited three sharp bands at 235, 292 and 387 nm (Fig. 2). Similarly, the receptor $\mathbf{R^2}$ shows three absorption bands at 235, 295 and 418 nm (Fig. 2). Upon gradual addition of the aqueous solution of Cu^{2+} ion in increasing concentration (0−100 μM), the bands of $\mathbf{R^1}$ and $\mathbf{R^2}$ at 387 and 418 nm, respectively, show significant decrease in the initial absorption intensity. Notably, both receptors $\mathbf{R^1}$ and $\mathbf{R^2}$ displayed a new absorption band centered at 460 and 486 nm, respectively, with increasing intensity which is attributed to the ground-state charge-transfer complexes $\mathbf{R^{1,2}}$–Cu^{2+} (Fig. 2). Appearance of well anchored two isobestic points centered at λ = 354 and 424 nm for $\mathbf{R^1}$ and λ = 375 and 445 nm for $\mathbf{R^2}$ are consistent to an equilibrium existence of receptors $\mathbf{R^1}$/$\mathbf{R^2}$ and copper complexes $\mathbf{R^{1,2}}$–Cu^{2+} in solution. Furthermore, a linear relationship was obtained from the absorption titration profiles for the plots [R = 0.9683 ($\mathbf{R^1}$) and 0.9992 ($\mathbf{R^2}$)] of measured $[1/(A-A_0)]$ at 460 nm for $\mathbf{R^1}$ and 486 nm for $\mathbf{R^2}$ as a function of $1/[Cu^{2+}]$ using the well known linear Benesi–Hildebrand expression, which indicates a ~1:1

stoichiometry complex formation between receptors **R**[1] or **R**[2] and Cu^{2+} ion (Fig. 2) in solution. Calculated association constants of **R**[1] and **R**[2] are $K_a = 31.4 \times 10^4$ and 68.5×10^5 M^{-1}, respectively.

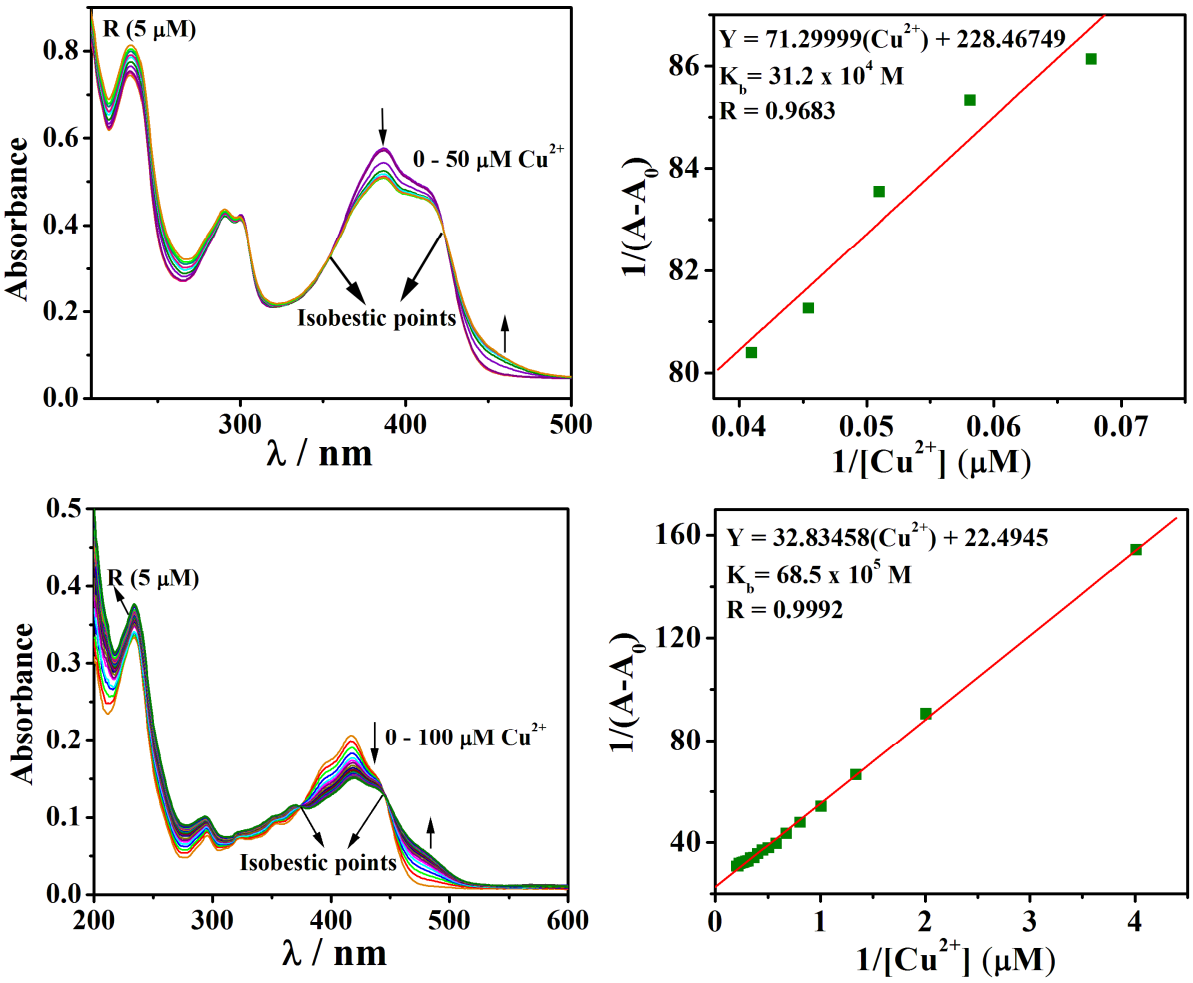


Fig. 2. Change in the absorption spectra of receptors **R**[1] (left top) and **R**[2] (left bottom) in the presence of increasing concentration (0−100 μM) of Cu^{2+} ions and their corresponding Benesi-Hilder Brand plots (right-top and bottom).

Notably, the addition of other metal cations such as Na^{+}, K^{+}, Mg^{2+}, Ca^{2+}, Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Cd^{2+} and Hg^{2+} did not alter the initial absorption spectrum of the receptors **R**^{1} and **R**^{2} significantly (Fig. 3). From these UV-vis studies, it is clear that receptors **R**^{1} and **R**^{2} show very high selective binding affinity in the ground state only for Cu^{2+} ion even in the presence of different other metal ions.

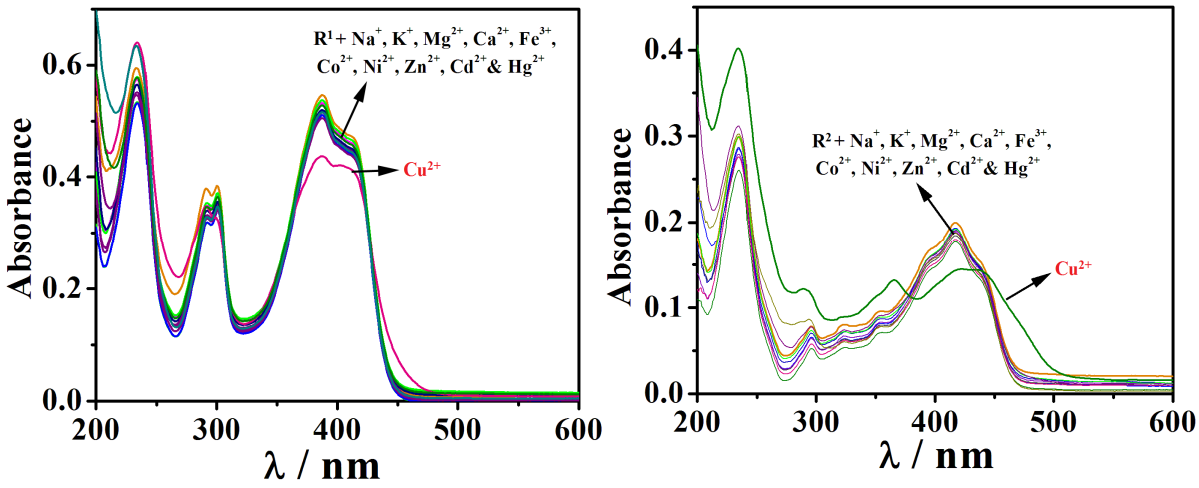


Fig. 3. Change in the absorption of receptors **R**^{1} (left) and **R**^{2} (right) upon mixing with different metal cations.

The fluorescence spectra of the receptors **R**^{1} and **R**^{2} (5 μM) exhibit a strong emission at 571 and 523 nm, respectively, in H_2O/CH_3CN (2:1, v/v) medium. Upon gradual addition of increasing amounts of aqueous Cu^{2+} solution (0−10 μM) to the solutions of **R**^{1} and **R**^{2}, the initial emission at 571 and 523 nm are quenched significantly (Fig. 4). This dramatic quenching of initial fluorescence intensity of **R**^{1} and **R**^{2} induced by Cu^{2+} ion is attributed to the reverse photo-induced electron transfer from pyrene-moiety to the phenolic-OH and hydrazide-N atoms due to the decreased in electron density upon the metal ion complexation.[28] Furthermore, the time resolved fluorescence study showed no changes in

life time of $\mathbf{R^2}$ (0.32 ns) upon the gradual titration with Cu^{2+}, which support that the observed fluorescence quenching follows static quenching mechanism *via* the ground state complex ($\mathbf{R^2}$–Cu^{2+}) formations (Fig. S8). The calculated Stern-Volmer binding constants (K_{sv}) are 2.29 × 10^4 and 25.56 × 10^5 M^{-1}, respectively. The job's plots of the fluorescence titration profiles of $\mathbf{R^1}$ and $\mathbf{R^2}$ with added Cu^{2+} revealed a 1:1 stoichiometry between $\mathbf{R^{1,2}}$ and Cu^{2+} species (Fig. 5).

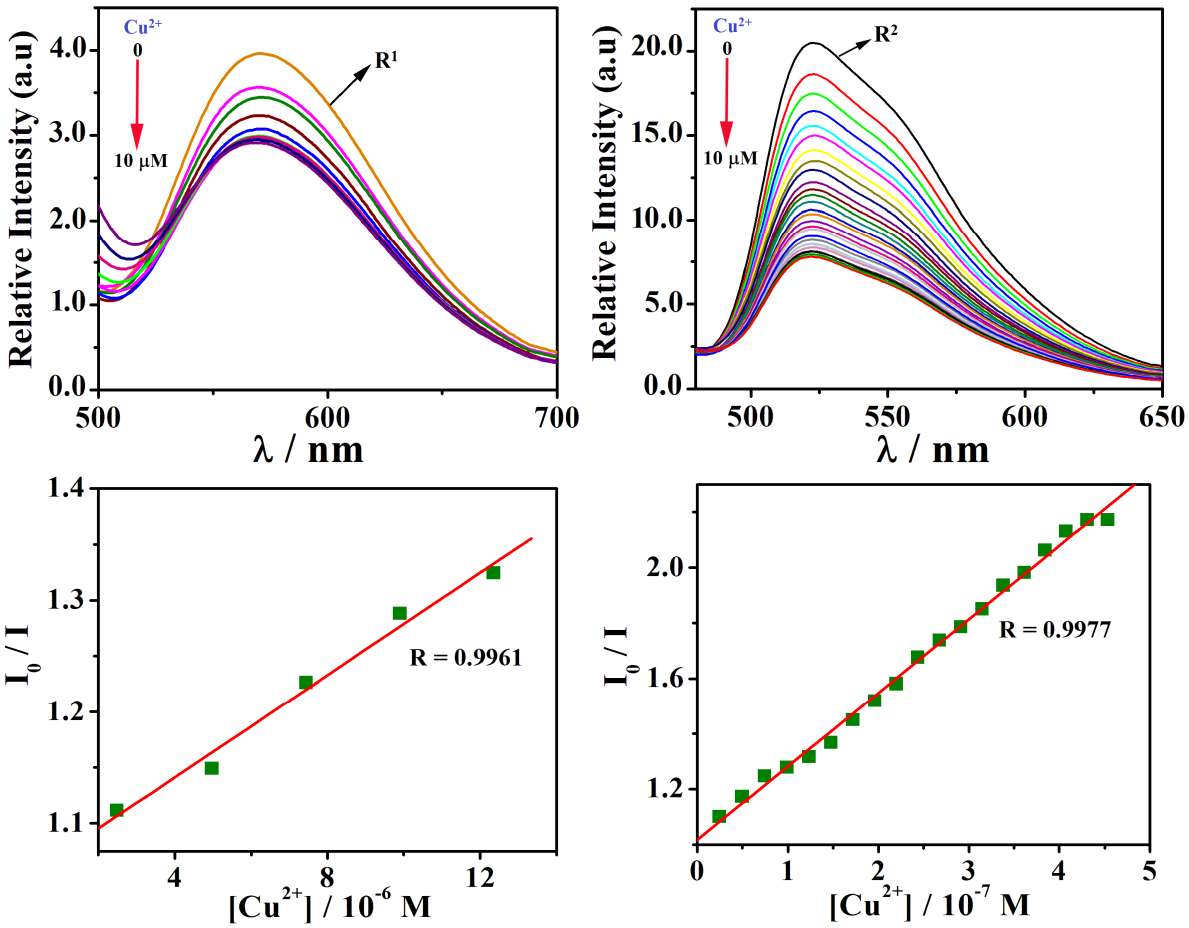


Fig. 4. Reduction in the initial fluorescence intensity (top) of $\mathbf{R^1}$ and $\mathbf{R^2}$ (5 μM) upon gradual increasing concentration of Cu^{2+} ion (0–12 μM) and their corresponding Stern-Volmer plots of $\mathbf{R^1}$ and $\mathbf{R^2}$ (bottom).

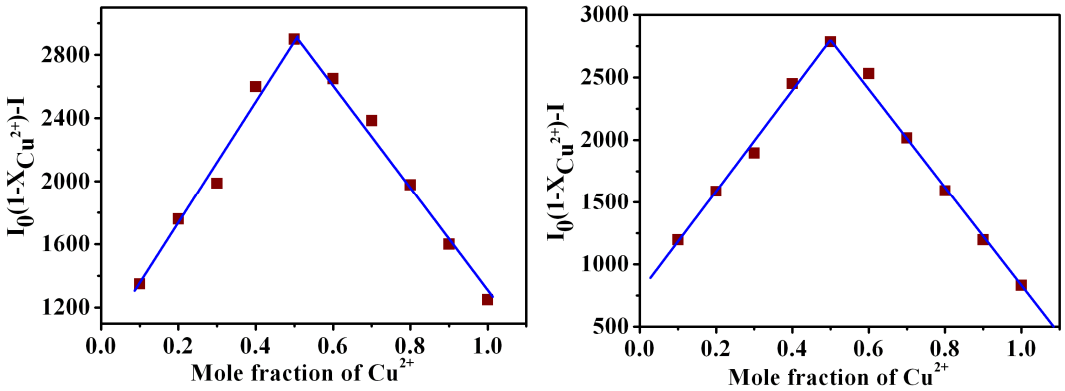


Fig. 5. Job's plots for the titration of receptors **R**^{1} (left) and **R**^{2} (right) with Cu^{2+} ion.

In order to prove the selectivity of receptors **R**^{1} and **R**^{2} towards Cu^{2+}, we carried the fluorescence titration experiments of **R**^{1} and **R**^{2} with other alkali (Na^{+}, K^{+}), alkaline-earth (Mg^{2+}, Ca^{2+}) and transition (Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Cd^{2+} and Hg^{2+}) metal ions. As shown in Fig. 6 and Fig. S9 only Cu^{2+} elicited a significant fluorescence quenching responses, while the other tested metal ions such as Na^{+}, K^{+}, Mg^{2+}, Ca^{2+}, Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Cd^{2+} and Hg^{2+} exhibited almost no fluorescence quenching response under the identical spectroscopic conditions as used for Cu^{2+}. Thus, receptors **R**^{1} and **R**^{2} could be used as highly selective fluorescence sensors for Cu^{2+} ion over other metal species in aqueous medium.

To corroborate the practical applicability of receptors **R**^{1} and **R**^{2} as selective fluorescence probes for Cu^{2+} ion, we carried out a competitive fluorescence titration study with other competing metal ions. As shown in Fig. 7 and Fig. S10, the initial fluorescence intensity of **R**^{1} and **R**^{2} did not changed significantly (red bars) upon mixing receptors **R**^{1} and **R**^{2} with one equivalent of different other metal cations (Na^{+}, K^{+}, Mg^{2+}, Ca^{2+}, Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Cd^{2+} and Hg^{2+}).

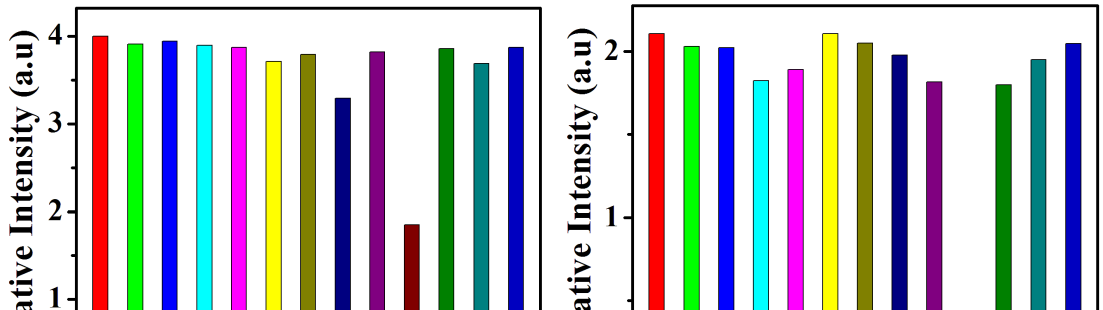


Fig. **6**. Change in the initial fluorescence intensities of receptors $\mathbf{R^1}$ (left) and $\mathbf{R^2}$ (right) (5 μM) in presence of 1.0 equiv of different metal cations in H_2O/CH_3CN (2:1, v/v) medium.

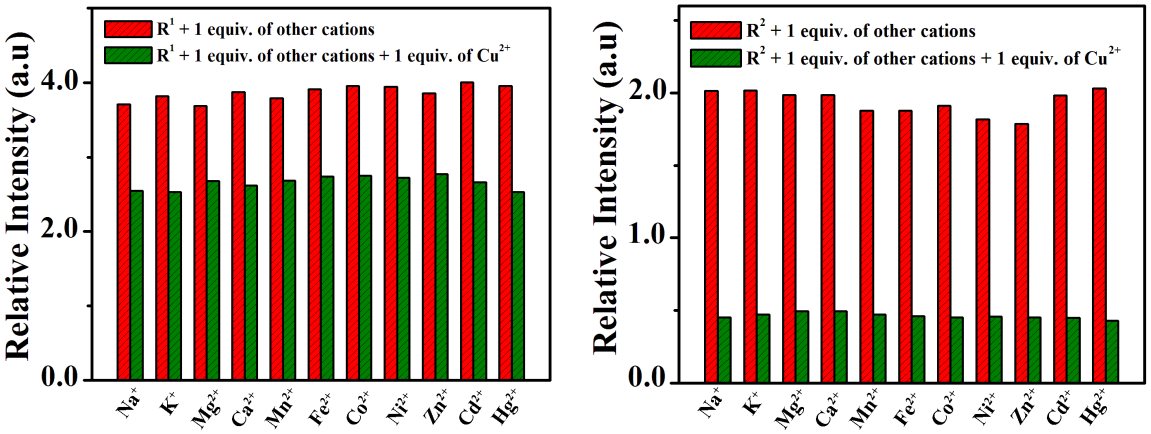


Fig. **7**. Competitive selective binding affinity of receptors $\mathbf{R^1}$ (left) and $\mathbf{R^2}$ (right) (5 μM) towards Cu^{2+} ions in the presence of 1.0 equiv of different metal cations in H_2O/CH_3CN (2:1, v/v) medium.

As far as real-time practical application is concerned, the sensing process of any receptor system must be a reversible one. To verify whether sensing process of $\mathbf{R^1}$ and $\mathbf{R^2}$ are reversible, one equivalent of ethylenediamine (en) solution was added into the solutions of $\mathbf{R^1}$ and $\mathbf{R^2}$ which were pre-incubated with one equivalent of Cu^{2+} solution. After the

addition of en solution, the initial emission intensity of $\mathbf{R^1}$ and $\mathbf{R^2}$ were almost recovered immediately from non-fluorescent $\mathbf{R^{1,2}}$–Cu^{2+} complex (Fig. 8). These results suggest that the high reversibility of $\mathbf{R^1}$ and $\mathbf{R^2}$ towards Cu^{2+} sensing and potential in application of real-time monitoring.

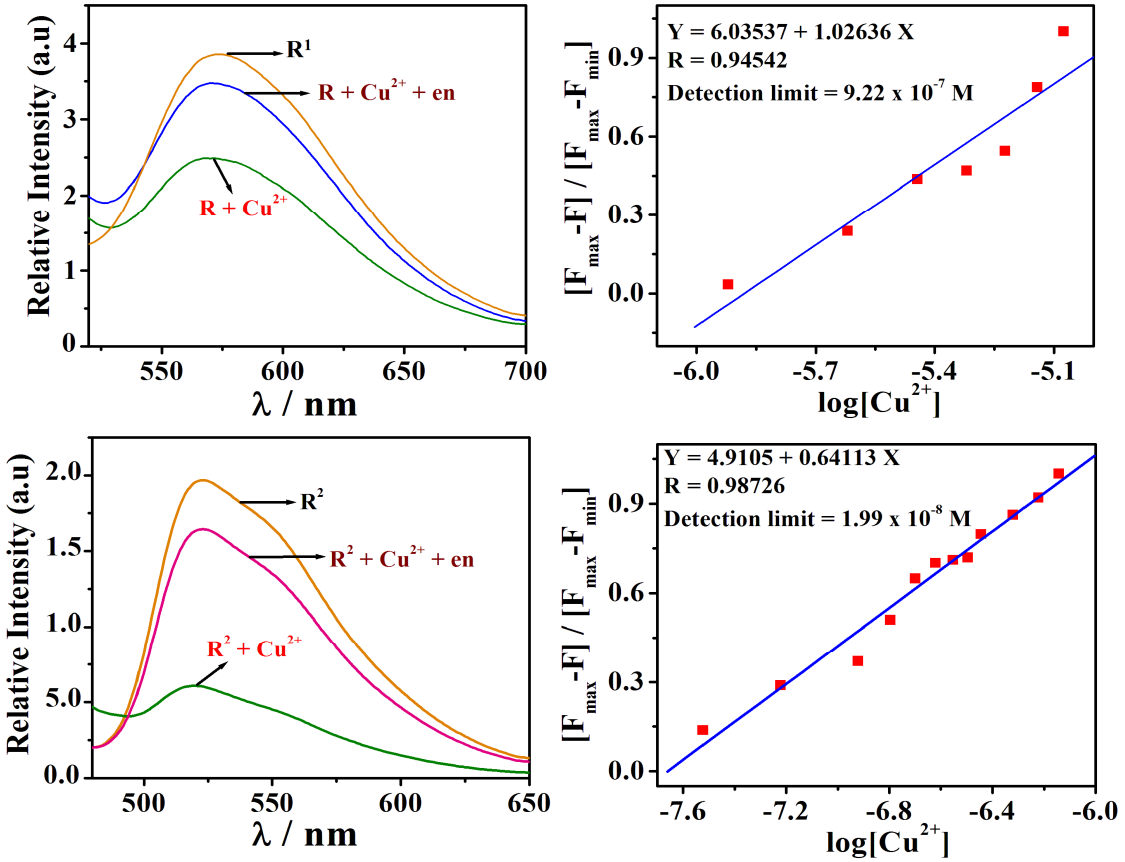


Fig. 8. Fluorescence spectra showing reversibility of Cu^{2+} (10 μM) towards receptors $\mathbf{R^1}$ and $\mathbf{R^2}$ (5 μM) (left) upon the addition of ethylenediamine (en) (10 μM) in H_2O/CH_3CN 2:1 (v/v) and detection limit plots (right).

In addition, the fluorescence titration profiles also demonstrates that the receptors $\mathbf{R^1}$ and

R² have the detection limits of 9.22×10^{-7} M (58.5 ppb) and 1.99×10^{-8} M (1.26 ppb) for Cu^{2+}, respectively, which is higher than that of reported Cu^{2+} chemosensors[29-30] and this level of detection limit is sufficient enough to sense Cu^{2+} ion even in the biological systems (Fig. 8).[31]

Bioimaging studies

Based on interesting photophysical properties of **R¹** and **R²** such as high sensitivity, selectivity and fast-responses toward Cu^{2+} ion, we further extended our study to evaluate their potential for application in imaging Cu^{2+} in cancer cell lines.

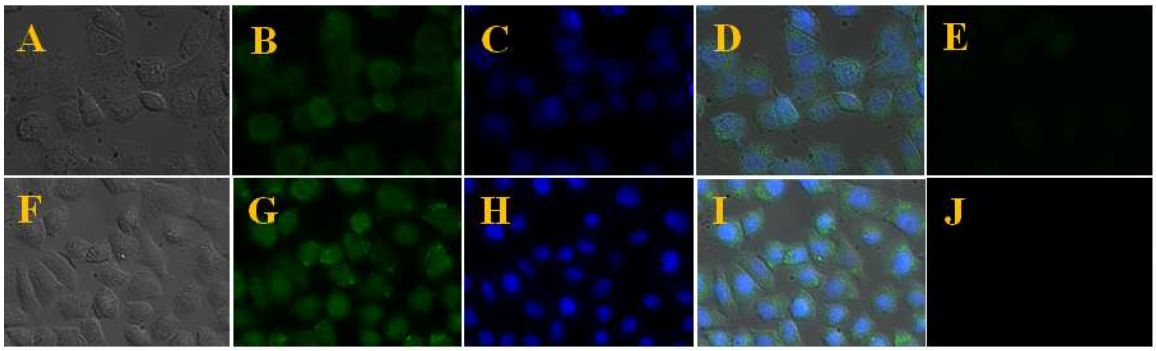


Fig. 9. Confocal fluorescence images of Cu^{2+} in HeLa cells (ApoTome [ZEISS] Fluorescence microscope). (A, F) Bright-field transmission images of HeLa cells. (B, G) Fluorescence images of HeLa cells incubated with **R¹** and **R²** (5 μM), respectively. (C, H) Hoechst 33342 stained fluorescence images of HeLa cells incubated with **R¹** and **R²** (5 μM), respectively. (D, I) Merged images of (B & C) and (G & H), respectively. (E, J) Cells supplemented with **R¹** and **R²** (5 μM) in the growth media for 0.5 h at 37 °C and then incubated with $CuCl_2$ (10 μM) for 1 h at 37 ºC.

The human cervical HeLa cancer cell lines incubated for 0.5 h at 37 °C with different concentrations of **R**^{1} and **R**^{2} (1.0 & 5.0 μM in H_2O/CH_3CN (2:1, v/v) buffered with HEPES, pH = 7.0) in a DMEM medium for 20 min at 37 °C and washed with a phosphate-buffered saline (PBS) (pH = 7.4) to remove excess of receptors **R**^{1} and **R**^{2}. Microscopic images showed bright fluorescence due to the accumulation of **R**^{1} and **R**^{2} within the cells (Fig. 9 and Fig. S11). The fluorescence was mostly localized in the cytoplasm of the cells. But in contrast, the subsequent staining of pre-incubated cells with Cu^{2+} (2.0 & 10.0 μM) for 1 h at 37 °C exhibited almost no fluorescence. These results suggest that receptors **R**^{1} and **R**^{2} are highly cell membrane permeable and can be used as bio-sensors to probe the intracellular Cu^{2+} concentration[32-36] and investigate its bioactivity in living cells.

Conclusions

In conclusion, two pyrene hydrazone-based fluorogenic chemosensors **R**^{1} and **R**^{2} have been designed and used them as selective, sensitive and reversible sensors for Cu^{2+} in aqueous medium. Based on the sensing studies, naphthaldehyde containing chemosensor **R**^{2} displays efficient sensitivity and selectivity towards Cu^{2+} than salicylaldehyde incorporated analogue **R**^{1}. This may be due to the presence of extended aromatic moiety in naphthalene ring, which makes more plausible PET mechanism than phenyl ring of salicylaldehyde. In addition, we further demonstrated that these new chemosensors can be utilized in live cell imaging of Cu^{2+} ion. The excellent detection limits 5.85 ppb (**R**^{1}) and 12.60 ppb (**R**^{2}) of these chemosensors would be useful in detection of trace quantity of Cu^{2+} in biological and environmental samples.[32-36] Competitive bioimaging studies

imply that the fluorescent probes (**R**[1] and **R**[2]) for intracellular imaging of Cu^{2+} ions with tuneable emission (green) will help in the understanding of biological processes at the molecular level.

Supporting information: Synthesis, characterization data of R^1 and R^2 including crystallographic data in CIF (CCDC-896663) and fluorescence titration plots.

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TOC

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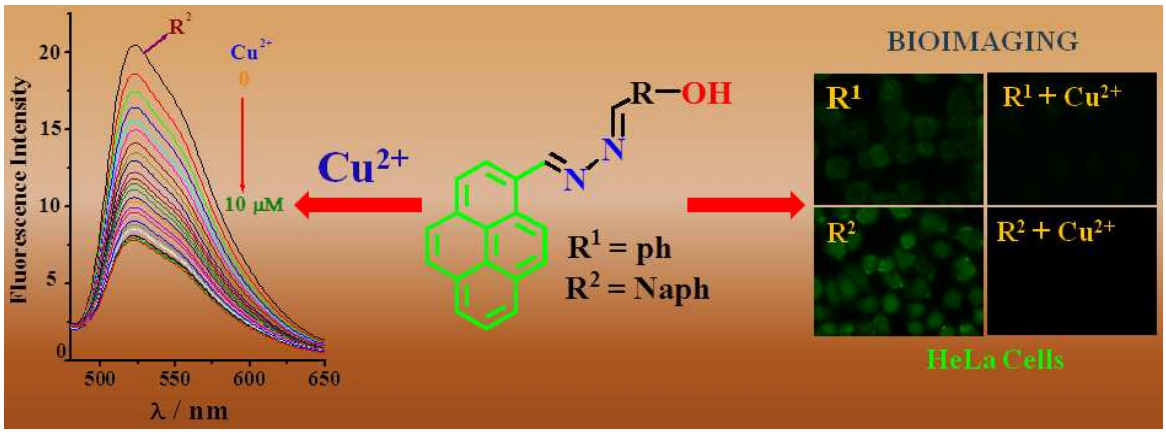

Synthesis and characterization of two pyrene-hydrazone based fluorescent sensors for Cu^{2+} with detection limit of ppb level have been presented. It is also demonstrated that these new chemosensors can be utilized in live cell imaging of Cu^{2+} ion.