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Highly Selective Probe Detects Cu^{2+} and Endogenous NO Gas in Living Cell

Balakrishnan Muthuraj,[†] Rohitas Deshmukh,[‡] Vishal Trivedi,*[,‡] and Parameswar Krishnan Iyer*[,†,§]

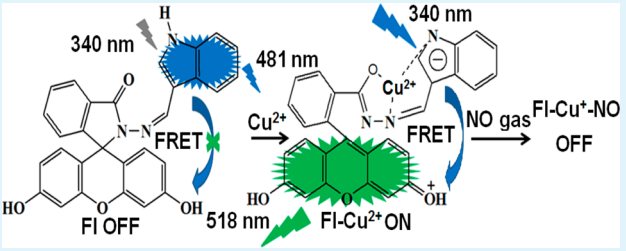
[†]Department of Chemistry, Indian Institute of Technology, Guwahati-781039, Assam, India

[‡]Malaria Research Group, Department of Biotechnology, Indian Institute of Technology, Guwahati, Assam-781039, India

[§]Centre for Nanotechnology, Indian Institute of Technology, Guwahati-781039, Assam India

Supporting Information

ABSTRACT: The rapid and highly sensitive detection of extremely short-lived nitric oxide (NO) gas generated *in vivo* by a water-soluble fluorescein derivative is developed. This assay system comprises of indole-3-carboxaldehyde functionalized fluorescein hydrazone (FI) assay which displays a typically high absorption at 492 and 620 nm in the presence of Cu^{2+} and also shows FRET induced fluorescence turn-on exclusively with Cu^{2+}. FI selectively detects Cu^{2+} *in vivo* and *in vitro* by the "turn-on" mechanism followed by fluorescence "turn-off" with NO gas generated by the lipopolysaccharide (LPS) action. The *in vivo* experiment performed in the cellular system indicates that FI loaded RAW264.7 cells showed bright fluorescence in the presence of Cu^{2+}, while other metals did not influence the FI fluorescence. In addition, the fluorescence of FI−Cu^{2+} was efficiently quenched by NO generated in macrophages through LPS stimulation. FI demonstrates characteristic "turn-on" behavior in the presence of Cu^{2+} via spirolactom ring-opening, while other metals such as Na^{+}, K^{+}, Ca^{2+}, Cr^{3+}, Mn^{2+}, Fe^{3+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Cd^{2+}, Hg^{2+}, and Ag^{+} did not influence FI fluorescence even at very high concentration. Further, the FI−Cu^{2+} complex fluorescence was not quenched with any anions or amino acids but totally quenched by NO and the paramagnetic nature of Cu^{2+} ion converted into the diamagnetic nature when reduced to Cu^{1+}. FI and the FI−Cu^{2+} complex are nontoxic to the cellular system and have high potential for biomedical applications.



KEYWORDS: *fluorescein, copper, NO gas, sensor, fluorometric, colorimetric*

INTRODUCTION

Copper, one of the foremost metals known to human and the third most plentiful transition metal[1] in the human physiology, is indispensable for carrying out several necessary processes in the human body as well as in the environmental cycle. Copper combines with proteins to produce enzymes that are associated with oxygen processing and also acts as a catalyst in body functions. At low concentrations, it is an essential trace nutrient for all organisms, but at high concentrations it is poisonous. Accumulation of a large excess of copper in the brain and the liver is highly toxic and causes Alzheimer, Parkinson, Prion, Menkes, and Wilson's disease.[2] Therefore, detection of copper at very low concentrations in the biological environment is very important. Similarly, nitric oxide (NO) gas, produced by nitric oxide synthases enzyme, is now recognized as a vital biological signaling molecule present in the human body and plays significant roles in several biological processes.[3,4] Although NO was identified as an environmental pollutant, it is also recognized now as a biologically relevant signaling molecule in cardiac functions, neurotransmission, vasodilation, etc. since this highly reactive free radical can diffuse through the cells easily.[5,6] The lifetime of NO is usually extremely short-lived and is dependent on the environmental conditions,[7,8] and nitrosated species such as S-nitrosothiols and N-nitrosamines are proposed to act as vehicles for NO storage and transport in biology.[9,10] In addition, NO can also reductively nitrosylate metals, thereby inducing one-electron reduction of the metal and nitros(yl)ation of a nucleophile by the resulting NO^{+} to form an E-NO species, where E can be either O or N or S.[11] There are few core requirements to design biologically useful fluorescent NO probes, such as water solubility, low toxicity, cell-membrane permeability, easy synthesis, and *in vivo* monitoring. Most importantly, the fluorescent probe must have the capability to detect NO specifically in a competitive environment due to the extremely short-lived nature of this species.[12] Fluorescein, a biologically friendly fluorophore, has high water solubility, high fluorescence quantum yield, and high molar extinction coefficient value.[13] It is used as a core moiety for several common fluorescent probes especially in eye care, tumor surgery,[14−17] etc. and more recently to detect metals, reactive oxygen, and nitrogen species.[6,12,18−21] Nontoxic fluorescein and its derivatives, such as aminofluorescein[22] and

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some high electron donor containing fluorescein moieties, are nonfluorescent, due to the high HOMO level of its electron-donating group. Hence, it can detect NO with very high fluorescence enhancement. Various other NO turn-on sensors, including o-diaminofluorescein, o-diaminonaphthalene, o-diaminocyanine, luminescent lanthanide complexes, and 5-amino-1-naphthonitrile, have been reported to detect NO.[23−27] A few other recently developed copper-based fluorescent probes provide direct and selective detection of NO both *in vitro* and *in vivo*.[28−34] Although rhodamine based turn-on copper sensors are reported to induce spirolactam ring-opening via Cu^{2+} coordination,[1,35−45] there are very few reports on turn-on copper sensors that can be utilized for imaging in living cells.[40,46−50] Hence, the development of efficient fluorescent sensors with potential applications in bioimaging and environmental research remains not only challenging but in most cases are also very difficult to develop.[51,52] In continuation to our efforts in developing new metal and biological sensors,[53−58] we report here the synthesis of a fluorescein-indole probe (FI) which is soluble in alkaline medium and can be used for the highly selective detection of copper and NO gas in living cells.

EXPERIMENTAL SECTION

Materials and Methods. All reagents and solvents were purchased from commercial sources, and the solvents used were of spectroscopic grade. UV−vis absorption spectra were recorded on a PerkinElmer Lambda-25 spectrometer. Fluorescence spectra were carried out on a Varian Cary Eclipse Spectrometer. A 10 mm × 10 mm quartz cuvette was used for solution spectra, and emission was collected at 90° relative to the excitation beam. Deionized water was obtained from the Milli-Q system (Millipore). ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were obtained with a Varian-AS400NMR spectrometer. Crystal data were collected with Bruker Smart Apex-II CCD diffractometer using graphite monochromated Mo−Kα radiation (λ = 0.71073 Å) at 298 K. Mass spectra were recorded on a Agilent Accurate-Mass Q-TOF LC/MS 6520, and peaks are given in m/z (% of basis peak). The X-Band Electron Paramagnetic Resonance (EPR) spectra were recorded on a JES-FA 200 EPR spectrometer, at room temperature. The cells were mounted on the glass slide in CC mount (Sigma) and observed under the 40× objective of fluorescence microscope eclipse 80Ti (Nikon), and images were captured using high resolution camera.

Synthesis of FI. The probe FI was synthesized by a reaction between fluorescein hydrazine and indole-3-carboxaldehyde in methanol containing acetic acid (see Figures S1 and S2 in the Supporting Information). The product was characterized by electrospray ionization mass spectrometry (ESI-MS), nuclear magnetic resonance (NMR) spectroscopy, and X-ray crystallography (see Figures S3 in the Supporting Information).

Preparation of Stock Solutions. The FI stock solution was prepared at the concentration of 1.0 × 10^{-3} mL^{-1} in 10 mL of CH_3CN. This stock solution was diluted to a desired concentration for each titration in a 3 mL cuvette having HEPES buffer.

Preparation of Cation and Anion Stock Solutions. Each inorganic metal salt and anions stock solutions were prepared at the concentration of 10.0 × 10^{-3} mL^{-1} in 5 mL of Milli-Q water. The stock solutions were diluted to the desired concentrations with Milli-Q water when needed.

Preparation of HEPES Buffer Solution for Titration Experiments. All the UV−visible and fluorescence titrations were performed in 10 mM HEPES buffer at pH 7.4 by using 4 M NaOH or 5 M HCl solution.

UV−Visible Spectra Titration of FI with Cu^{2+}. FI and a series of FI−Cu^{2+} in HEPES buffer solutions with different concentrations of FI and Cu^{2+} in 1:1 ratios were stored for 0−12 h at room temperature. Absorption peak was observed at 340 nm for free FI solution. Interestingly, an absorption peak was observed at 492 nm for all these solutions, and their absorption intensities increased rapidly as the concentration of FI−Cu^{2+} increased from (0 μM to 35 μM) in HEPES buffered solution. This result indicates that FI can detect Cu^{2+} at very low micromolar levels and produce a fluorescence signal after the complex has been stored for some time.

Fluorescence Titration of FI with Cu^{2+}. A series of FI−Cu^{2+} solutions in HEPES buffer solutions (pH 7.4) with different concentrations of FI and Cu^{2+} ratios were stored for 0−12 h at room temperature. Interestingly, an emission peak was observed at 518 nm (λ_{ex} = 465 nm) for all these solutions, and their fluorescence intensities increased rapidly as the concentration of FI (3 μM) with Cu^{2+} (0 μM to 9 μM) in HEPES buffered solution. This result indicates that FI can detect Cu^{2+} at very low nanomolar levels and produce a fluorescence signal after the mixture was stored for prolonged time.

Fluorescence Titration of FI with Cu^{2+}. A series of FI (10 μM) solutions in HEPES:CH_3CN, 6:4, v/v; pH 7.4, solutions with different amounts of copper (0−70 μM) were stored for 0−12 h at room temperature. Interestingly, an emission peak was observed at 518 nm (λ_{ex} = 340 nm) for all these solutions, and their fluorescence intensities increased rapidly as the formation of FI−Cu^{2+} in HEPES:CH_3CN, 6:4, v/v; pH 7.4 buffered solutions.

Fluorescence Titration of FI−Cu^{2+} with NO Solution. Fluorescence quenching titration of FI+Cu^{2+} was done in the presence of different concentrations of NO (0−100 μL from saturated solution of NO in CH_3CN) in HEPES buffered solution at pH 7.4. The gradual addition of NO by syringe to the FI+Cu^{2+} complex in a 3 mL closed cuvette shows that the fluorescence peak at 518 nm was completely quenched at a final volume of 100 μL of NO solution. The same fluorescent quenching titration of FI+Cu^{2+} with anions like F^-, Cl^-, Br^-, I^-, N_3^-, NO_2^-, NO_3^-, CH_3COO^-, ClO_4^-, $H_2PO_4^-$, HSO_4^-, and S^{2-} was performed in HEPES buffered solution at pH 7.4.

Fluorescence Imaging of Cu^{2+} and NO in Cells. The mouse macrophage cell line RAW 264.7 was grown in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma, St. Louis, MO, USA), containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin antibiotic (100 units/mL penicillin and 100 μg/mL streptomycin sulfate) at 37 °C in a humidified incubator containing 5% CO_2. A day before the experiment, 10^5 cells were plated on sterilized square coverslips (12 mm) in a total volume of 1.5 mL in 35 mm cell culture dishes. Cells were allowed to adhere to the coverslip. Thereafter, cells were incubated with fluorescent FI probe (100 μM) overnight in complete media. Cells were washed 2 times with sterile cell culture grade PBS and treated with Cu^{2+} (50 μM) for 1 h at 37 °C at 5% CO_2 in 1.5 mL of serum free media. To detect intracellular nitric oxide, cells were treated with heat killed bacteria (LPS) in 1:25 ratio in serum free media for different times (0−4 h) at 37 °C at 5% CO_2. After the particular time point, cells were washed with sterile PBS and fixed with 2% paraformaldehyde in PBS. The cells were mounted on the glass slide in CC mount (Sigma) and observed under the 40× objective of fluorescence microscope eclipse 80Ti (Nikon), and images were captured using high resolution camera. Files were opened in Adobe Photoshop 7.0, and gray levels were adjusted by using the auto level command with a black and white clip set to 0%. Images were cropped again and scaled for final display.

Fluorescence Imaging of Probe FI with Cu^{2+} Ion in Living Cells. RAW 264.7 was loaded with FI and treated with different metals (Cu^{2+}, Co^{2+}, Cd^{2+}) for 1 h at 37 °C. Post-treatment, cells were fixed and mounted in a CC mount (Sigma) and observed under the 40× objective of fluorescence microscope eclipse 80Ti (Nikon), and images were captured using high resolution camera.

Fluorescence Imaging of FI+Cu^{2+} Complex with Endogenous Generated NO Gas. Fluorescence imaging of FI−Cu^{2+} in the presence of RAW 264.7 cells was performed both in the presence and absence of *in vivo* generated NO gas. RAW 264.7 is loaded with FI (100 μM) overnight in complete media and then incubated with Cu^{2+} for 1 h at 37 °C to form an intracellular FI−Cu^{2+} complex. Afterward, cells were treated with heat killed *E. coli* (1:25) to generate intracellular NO and fluorescence signal of the FI−Cu^{2+} complex and was monitored for different time periods (1−3 h). Generation of

NO causes fluorescence quenching of FI−Cu^{2+} in a time-dependent manner with a complete quenching within 2 h.

Cellular Toxicity Evaluation of FI. MTT assay is used to test the cytotoxicity of FI as described with slight modifications.[59] Ten thousand J774A.1 cells were seeded overnight in a 96 well plate in 0.2 mL of serum containing media (complete media). Cells were treated with different concentration of FI (0−800 μM) in serum free media for 48 h at 37 °C with 5% CO_2. Post-treatment, cells were washed twice with PBS and incubated with 100 μL of MTT (0.5 mg/mL) for 4 h at 37 °C with 5% CO_2. Then, MTT solution was removed, and formazan crystals were dissolved in 100 μL of cell culture grade DMSO. The optical density was determined using a spectrophotometer at 570 and 660 nm. Cells treated with serum free media were considered as 100% viable, and the result of treatment is expressed as the percent survival in comparison to the control.

RESULTS AND DISCUSSION

The fluorescein-indole probe (FI) presented here (Figure 1a) offers advantages such as the ease in synthesis, water solubility, a highly efficient homogeneous assay system with very low detection limit for copper and NO gas both *in vitro* and *in vivo* generated endogenously. This probe also presents a convenient mix and detects strategy with rapid output, representing a simple and continuous strategy to monitor the extremely small and short-lived NO gas evolved inside the cell. We observe a coordination induced Förster resonance energy transfer (FRET) which turns-on the FI in the presence of Cu^{2+} (Figure 1b) from a nonfluorescent state. This probe possesses a donor (indole-3-carboxaldehyde) and an acceptor fluorophore (xanthene) (Figure 1a), and the Cu^{2+} induces FRET between these pairs intramolecularly. The synthesis, characterization, and single crystal X-ray structure of FI is presented in Figures S1−S3 (SI).

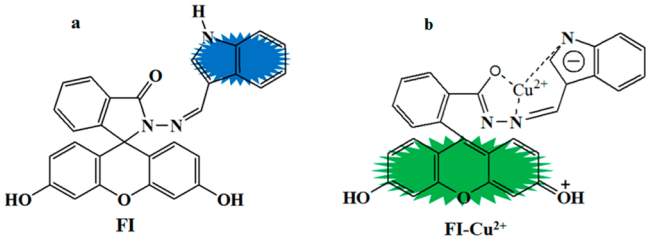

Figure 1. (a) Nonfluorescent probe FI and (b) FI+Cu^{2+} highly fluorescent complex.

A solution of FI (50 μM) in 10 mM HEPES (pH 7.4) buffer has an absorbance maxima at 340 nm (Figure 2a blue line), corresponding to the indole moiety. On titrating Cu^{2+} with FI, new absorbance peaks at 492 and 620 nm (weak) are observed (green lines) which represents the open spirolactam ring form of the xanthene moiety. Such a huge 152 nm Cu^{2+}-induced absorption shift could be attributed to the high conjugation and planarity of the indole moiety of FI with the binding sites which favors maximum negative charge distribution of the deprotonated receptor in the presence of Cu^{2+}.[60] FI in the presence of other cations did not show any spectral changes (Figure 2b).

The selective binding of FI (3 μM) with Cu^{2+} ions was studied using fluorescence spectroscopy in aqueous HEPES buffer (10 mM, pH 7.4) (Figure 3). FI contains two fluorophore units, and we have studied the binding event of FI at two different excitation wavelengths corresponding to the excitation wavelength of the xanthene unit (465 nm) and the indole unit (340 nm), respectively. When excited at 465 nm, FI did not show any emission in the range of 500 to 600 nm (Figure 3a, red line) indicating that in the absence of metal ions, FI remains in the spirolactam form (Figure 1a). Addition of Cu^{2+} to the FI induces a significant turn-on fluorescence response at 518 nm, with a highly green fluorescence (Figure 3a, Figure 4a). The apparent binding constant for the formation of the respective complexes were evaluated using the Benesi−Hildebrand (B−H) plot and was estimated to be 1.19×10^4 M^{-1} by the UV−vis spectral changes at 492 nm (Figure S4). It was also observed (Figure 3a) that the metal−ligand binding induced ring-opening of FI and the generation of xanthene moiety was highly selective toward Cu^{2+} ions only, whereas no spectral changes occur in the presence of other metal ions like Na^+, K^+, Ca^{2+}, Cr^{3+}, Mn^{2+}, Fe^{3+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Cd^{2+}, Hg^{2+}, and Ag^+ (Figure 3b).

Moreover, the fluorescence intensity of FI was enhanced with the addition of Cu^{2+} ions when excited at 340 nm, which further confirmed that the probe FI exhibited a high sensitivity only toward Cu^{2+} (Figure 4a). Upon sequential addition of Cu^{2+}, the indole emission signal intensity at 481 nm decreased, and a strong emission signal corresponding to ring-opened fluorescein appeared at 518 nm. This indicates that a highly efficient coordination induced FRET process in the presence of Cu^{2+} produces an intense fluorescein-based green emission (Inset: Figure 4a); i.e., intramolecular energy transfer from the indole donor to the xanthene acceptor is due to the ring-opening resulting in an increase of overlap integral between indole and xanthene moiety.[61] This overlap between emission of indole and absorption of ring-opened fluorescein unit strongly supports the high energy transfer efficiency and the

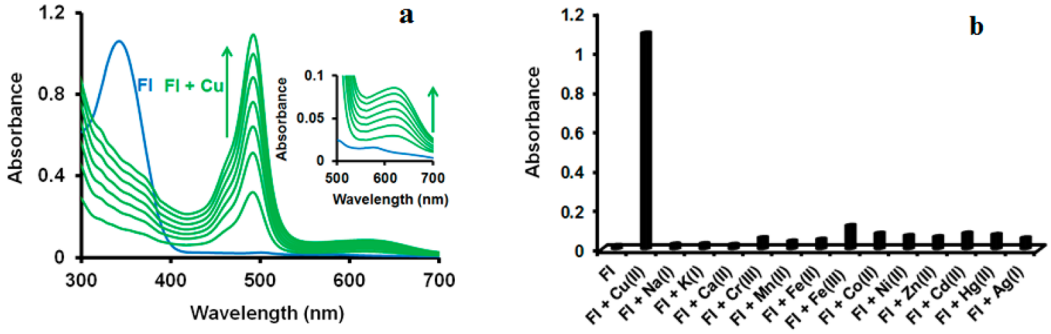

Figure 2. (a) UV−vis spectra of FI (blue line), FI with Cu^{2+} (1:1) (5−35 μM) shows absorbance at 492 and 620 nm (weak) at pH 7.4 (HEPES buffer). (b) Bar diagram of UV−vis changes observed in the peak of **FI** (30 μM) in the presence of various metal ions like Na^+, K^+, Ca^{2+}, Cr^{3+}, Mn^{2+}, Fe^{3+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Hg^{2+}, Ag^+ (150 μM).

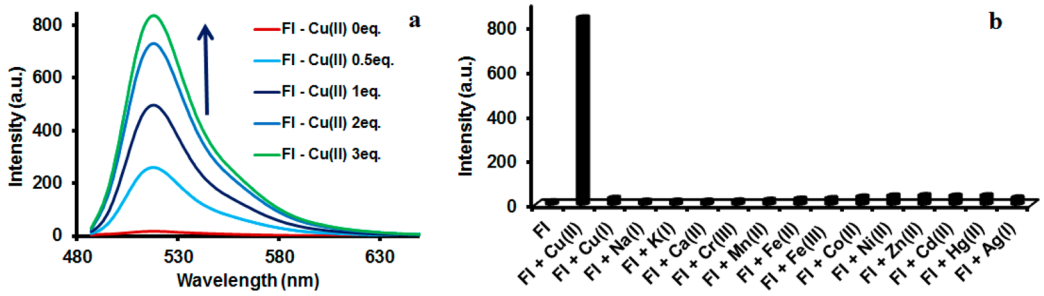

Figure 3. (a) Fluorescent titration spectra of FI (3 μM) in the presence of different concentrations of Cu^{2+} (9 μM) in HEPES buffered solution at pH 7.4. λ_{ex} = 465 nm, λ_{em} = 518. (b) Bar diagram of fluorescence changes observed in the fluorescence peak of FI in the presence of various metal ions like Na^{+}, K^{+}, Ca^{2+}, Cr^{3+}, Mn^{2+}, Fe^{3+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Hg^{2+}, Ag^{+} (30 μM).

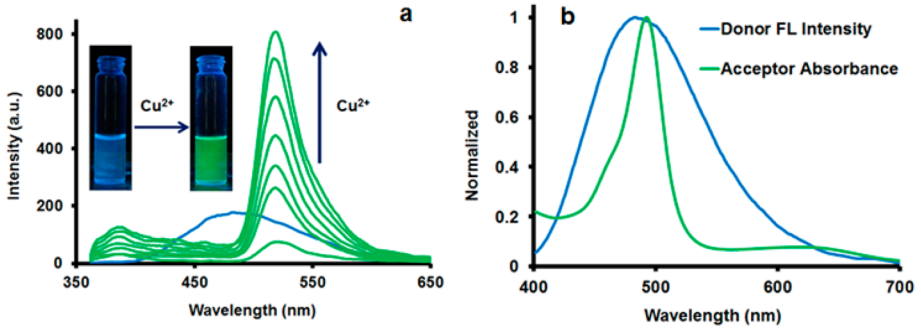

Figure 4. (a) Fluorescence spectra of FI (10 μM) with Cu^{2+} (0–70 μM) in 10 mM HEPES:CH_3CN, 6:4, v/v; pH 7.4, λ_{ex} = 340 nm, Inset: FI in the absence (blue) and the presence (green) of Cu^{2+} upon irradiation using a UV-lamp at 365 nm. (b) Spectral overlap (FRET) between emission of indole (blue) and absorbance of xanthene (green).

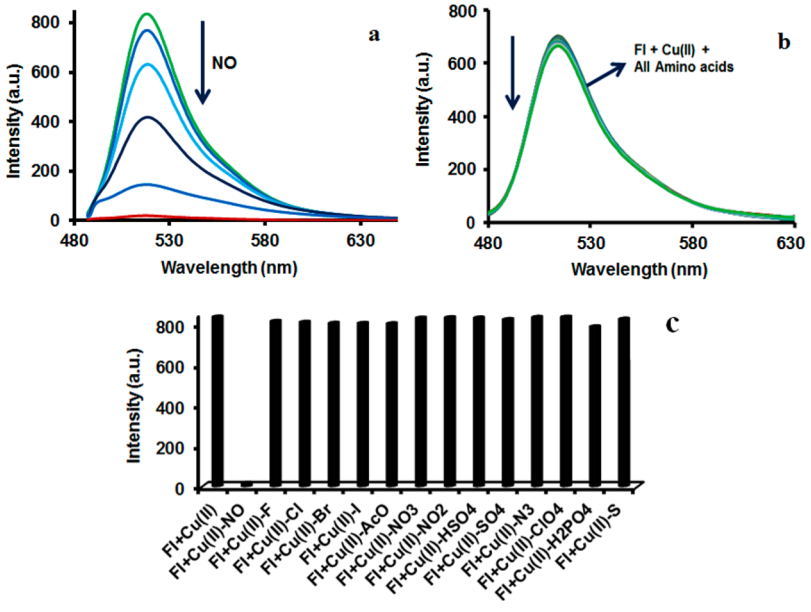

Figure 5. (a) Fluorescent titration spectra of FI (3 μM) + Cu^{2+} (9 μM) in the presence of different concentrations of NO (5 × 20 μL) in HEPES buffered solution at pH 7.4. λ_{ex} = 465 nm, λ_{em} = 518. (b) Fluorescent titration spectra of FI (3 μM) + Cu^{2+} (9 μM) in the presence of different amino acids (100 μM) in HEPES buffered solution at pH 7.4. (c) Fluorescence spectra of FI+Cu^{2+} in HEPES buffered (pH 7.4) solution in the presence of other anions (F^{-}, Cl^{-}, Br^{-}, I^{-}, N_3^{-}, NO_2^{-}, NO_3^{-}, CH_3COO^{-}, ClO_4^{-}, $H_2PO_4^{-}$, HSO_4^{-} and S^{2-}) (180 μM).

proof of FRET mechanism in aqueous buffer medium (10 mM HEPES/CH_3CN, 6:4, v/v; pH 7.4). Furthermore, it was confirmed that the Cu^{2+} sensing behavior of FI remains largely unaffected in the pH range of 6–14 extending the application potential of FI in biology (Figure S5). The detection limit (3σ/k) of Cu^{2+} by FI was determined to be 22.2 nM (Figure S6),[62] much lower than the permissible Cu^{2+} level in drinking water (31 μM) prescribed by the World Health Organization (WHO).[63]

It was further observed that this highly fluorescent FI+Cu^{2+} complex was completely quenched upon gradual addition of NO (Figure 5a) (0–100 μL from the saturated NO stock solution in CH_3CN) which may either be due to nitrosation by NO or fluorophore displacement mechanism by NO resulting

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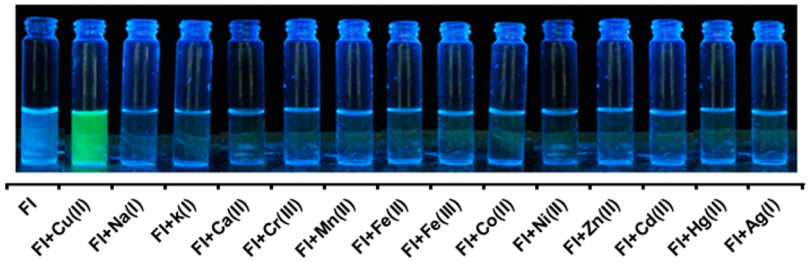

Figure 6. (a) Color and fluorescent changes of FI (30 μM) upon the addition of various metal ions (5 equiv) (Na^+, K^+, Ca^{2+}, Cr^{3+}, Mn^{2+}, Fe^{3+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Hg^{2+}, Ag^+) in HEPES-buffered solution (pH 7.4, 10 mM).

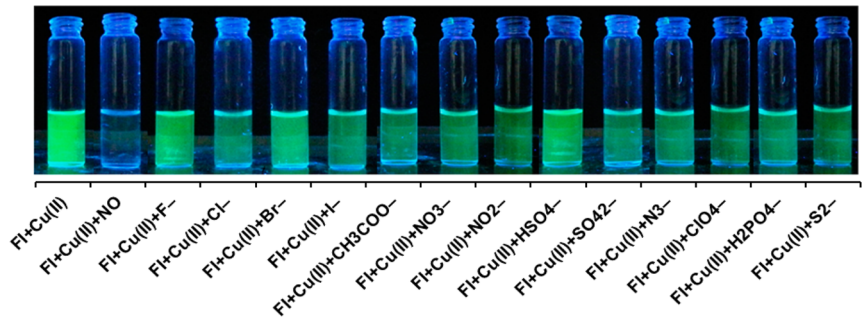

Figure 7. Color and fluorescent changes of FI+Cu^{2+} (30 μM) upon the addition of various anions (6 equiv) (F^-, Cl^-, Br^-, I^-, N_3^-, NO_2^-, NO_3^-, CH_3COO^-, ClO_4^-, $H_2PO_4^-$, HSO_4^-, SO_4^{2-}, S^{2-} and 100 μL of NO) in HEPES-buffered solution (pH 7.4, 10 mM).

in the fluorescence quenching event (Figure S7).[18−21,28−34] The detection limit (3σ/k) of NO for FI+Cu^{2+} was determined to be 13.8 μL (Figure S8).[62] The fluorescence response of FI +Cu^{2+} remained unaffected in the presence of amino acids or other anions like F^-, Cl^-, Br^-, I^-, N_3^-, NO_2^-, NO_3^-, CH_3COO^-, ClO_4^-, $H_2PO_4^-$, HSO_4^-, and S^{2-} (Figure 5b, Figure 5c).[64] The paramagnetic nature of Cu^{2+} ion in the FI +Cu^{2+} is converted to diamagnetic in the presence of NO when reduced to Cu^{1+} and confirmed by EPR spectra (Figure S9).[28]

Colorimetric Sensing Ability of FI toward Cu^{2+}. The colorimetric sensing ability of FI was investigated by adding various cations (Na^+, K^+, Ca^{2+}, Cr^{3+}, Mn^{2+}, Fe^{3+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Hg^{2+}, Ag^+) to an aqueous solution (HEPES buffer at pH 7.4) of sensor FI. When 5 equiv of Cu^{2+} was added to the solution of FI (30 μM), the sensor responded with a dramatic color change from light blue to bright green (Figure 6). In the corresponding UV−vis spectrum, the absorption appeared at 492 and 620 nm at pH 7.4 (Figure 2). In contrast when adding cations like Na^+, K^+, Ca^{2+}, Cr^{3+}, Mn^{2+}, Fe^{3+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Cd^{2+}, Hg^{2+}, and Ag^+ to solutions of the sensor FI, no significant color or spectral changes were observed.

Colorimetric Sensor of FI−Cu^{2+} toward NO Gas. The colorimetric sensing ability of the FI−Cu^{2+} complex was investigated by adding various anions such as F^-, Cl^-, Br^-, I^-, NO, N_3^-, NO_2^-, NO_3^-, CH_3COO^-, ClO_4^-, $H_2PO_4^-$, SO_4^{2-}, HSO_4^-, and S^{2-} to aqueous solution (HEPES buffer at pH 7.4) of sensor FI−Cu^{2+}. When a 100 μL saturated solution of NO was added into the solution of FI−Cu^{2+} (30 μM), the sensor responded with a dramatic color change from green to colorless (Figure 7) rapidly. In contrast when anions F^-, Cl^-, Br^-, I^-, N_3^-, NO_2^-, NO_3^-, CH_3COO^-, ClO_4^-, $H_2PO_4^-$, SO_4^{2-}, HSO_4^-, and S^{2-} were added to the solution of FI−Cu^{2+}, no significant color changes were observed.

Cellular Toxicity of FI. The cellular toxicity of FI was evaluated in J774A.1 cells using MTT assay. Cells exposed to different concentrations of FI for 48 h exhibit no significant change in viability up to the concentration 500 μM (~15% at 500 μM), but above this it gives dose-dependent toxicity toward macrophage J774A.1 cells (Figure S10).

FI Detects Cu^{2+} Ion in Living Cells. After performing the cytotoxicity studies, the imaging ability of FI for intracellular Cu^{2+} has also been confirmed in an experiment performed with RAW 264.7 cells. FI is specifically observed responding to the Cu^{2+} present in the cell environment. During Cu^{2+} toxicity studies in aquatic fauna, the transition metals accumulate within the intracellular storage system of the cell.[65] In our experiments, the cells were loaded with FI overnight and treated with Cu^{2+} (50 μM) for 1 h to explore the suitability of FI in detecting Cu^{2+} present inside the cell. FI loaded cells are healthy and nonfluorescent, and treatment with Cu^{2+} causes a time dependent change in intracellular fluorescence with a maximum fluorescence at 1 h (Figure 8). No significant level of oxidative stress or change in cellular morphology was observed in cells treated with Cu^{2+}, indicating no role of such factors in modulating the fluorescence signal of FI inside the cell. Besides Cu^{2+}, other metals (only Co^{2+} and Cd^{2+} presented) did not show a significant change in fluorescence inside the cell (Figure

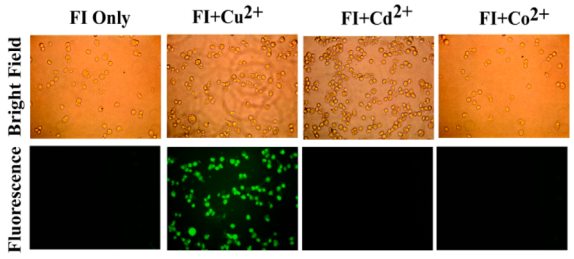

Figure 8. Fluorescence imaging of FI in RAW 264.7 cells in the presence and absence of Cu^{2+} ion. RAW 264.7 loaded with FI and treated with different metals (Cu^{2+}, Cd^{2+}, and Co^{2+}) for 1 h at 37 °C.

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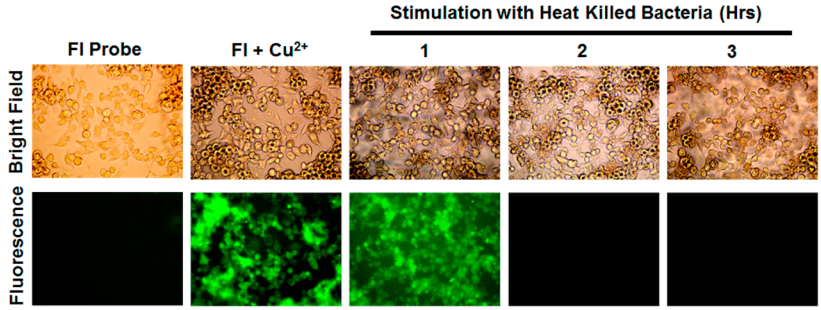

Figure 9. Fluorescence imaging of the FI−Cu^{2+} complex in RAW 264.7 cells in the presence and absence of *in vivo* NO gas using heat killed *E. coli* (LPS) (1:25) to generate intracellular NO gas. Incubated at 37 °C, the fluorescence signal of the FI−Cu^{2+} complex with heat killed *E. coli* (LPS) was monitored for different time periods (1−3 h). Complete quenching of FI−Cu^{2+} was observed after 2 h incubation due to NO gas liberated from heat killed *E. coli* (LPS).

8). Hence, imaging inside the live cells supports the *in vitro* fluorescence spectroscopy data and confirms suitability of FI to selectively detect intracellular Cu^{2+} (Figure 8).

Fluorescence Imaging of FI−Cu^{2+} in Macrophage To Detect NO Generated through LPS Stimulation. Further, the FI−Cu^{2+} complex can specifically detect NO gas generated endogenously, and as a result quenching of the FI−Cu^{2+} fluorescence occurs *in vivo*. Macrophage exposed to heat killed bacteria (lipopolysaccharide-LPS) causes production of intracellular NO through activation of iNOS.[66] Employing such an *in vivo* tool, we tested the suitability of the FI−Cu^{2+} complex as a NO sensor inside the cell. Macrophages RAW264.7 were loaded with FI and then treated with Cu^{2+} (50 μM) for 1 h to form the FI−Cu^{2+} complex as evidenced by an increase in fluorescence. Subsequently, the cells were treated with LPS to stimulate the production of endogenous NO gas. Production of endogenous NO quenches the fluorescence of the FI−Cu^{2+} complex in specific time and causes complete reduction of fluorescence intensity (Figure 9). FI loaded cells treated with individual metals or LPS alone did not cause any change in fluorescence. Hence, FI demonstrates the potential for detecting and imaging intracellular Cu^{2+}, and the resulting FI−Cu^{2+} complex can be applied to detect and monitor endogenous NO gas which is extremely short-lived and generally produced in incredibly low concentrations. FI or FI−Cu^{2+} complexes are nontoxic and have immense potential for future biomedical applications to measure Cu^{2+} accumulation or release of NO in biological systems.

CONCLUSION

In summary, we have developed a highly sensitive and biocompatible probe FI which selectively binds with Cu^{2+} ions and "turns-on" the response in optical and fluorescence spectra *in vivo* and *in vitro*. The new fluorescent Cu^{2+} sensor probe induces FRET based coordination between its two fluorophores donor indole and the acceptor Cu^{2+} bound xanthene moiety of FI and also facilitates imaging within the cells. Successive ring-opening of the spirolactam moiety in the presence of Cu^{2+} ion makes possible the naked eye detection via drastic color change and emission enhancement. The detection limit for Cu^{2+} was found to be 22.2 nM, which is much lower than the permissible Cu^{2+} concentration in drinking water as per the World Health Organization (WHO). FI selectively detects Cu^{2+} *in vivo* and *in vitro* by the "turn-on" mechanism followed by fluorescence "turn-off" in the presence of NO gas generated by the lipopolysaccharide (LPS) action. The *in vivo* experiment performed in the cellular system indicates that FI loaded RAW264.7 cells showed bright fluorescence in the presence of Cu^{2+}, while other metals did not influence the FI fluorescence. The complex formed between FI and Cu^{2+} is dissociated only in the presence of NO and remains unaffected in the presence of other anions and amino acids, which makes the FI−Cu complex a highly selective and sensitive sensor for NO *in vitro* and *in vivo* studies.

ASSOCIATED CONTENT

Supporting Information

Synthesis, spectroscopic studies, imaging results, and experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Authors

*E-mail: vtrivedi@iitg.ernet.in (V.T.).
*E-mail: pki@iitg.ernet.in (P.K.I.).

Notes

The authors declare no competing financial interest.

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