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ARTICLE

Chromogenic and fluorogenic detection of copper ions in the solution and intracellular media

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The development of a fluorescent sensor for the trace detection of the metallic contamination in the ecosystem and biological media have been a demand to reduce the health risk and environmental protection. Herein, we report the synthesis of 2-formyl-5-picolinyl-substituted rhodamine B derivative through Schiff base formation for the trace detection of the copper ions in the aqueous organic media as well as inside the live cells via bioimaging experiments. The ligand was successfully employed to detect the minute copper level ratiometrically through the chromogenic and fluorogenic response. The detection limit of the sensor was found to be 0.54×10^{-9} mol/L using 3 σ/slope relations. The reversible behavior of the ligand copper complex was determined using sulfide ions that regenerate the entire native properties of the ligands by snatching copper from the complex. The bioimaging experimental results represent the efficient membrane permeability of ligands with the minimum level of toxicity in the measured range found by the MTT assay. The appearance of bright red fluorescence from the BHK-21 (hamster kidney fibroblast) cells upon treatment of the ligand incubated cells with the copper ions represents the utility of the proposed sensor for the copper detection probe in the intracellular media.

KEYWORDS

bioimaging, chromogenic, copper sensor, cytotoxicity, fluorogenic, imine

1 | INTRODUCTION

The design and synthesis of artificial sensors for biologically important metal ions attracts the attention from the analytical chemists for the fast, accurate, reproducible, and selective determination of various species due to their fundamental roles in a wide range of chemical, environmental, and biological processes involved in multidisciplinary fields including life science, environmental, agricultural, industrial, medicinal analysis, and biotechnology.[1]

Copper (II) ion, being the third adequate element in the human body among the essential metals following iron and zinc, plays crucial roles in many fundamental physiological processes in organisms ranging from bacteria to mammals.[2] Copper ions also catalyze the formation of reactive oxygen species (ROS) that can damage lipids, nucleic acids, and proteins.[3] It is well known that it serves as a catalytic cofactor for a variety of metalloenzymes including superoxide dismutase, cytochrome *c* oxidase, and tyrosinase, and its homeostasis is critical for the metabolism and development of living organisms.[4] Copper-catalyzed enzymes regulate several body functions including skin pigmentation via melanin transformation, facilitate the formation of crosslinks in collagens, elastin, and involved in repair of connective tissues.[5] Unregulated overloading of copper neuronal cytoplasm can be toxic and can cause oxidative stress and

disorders associated with neurodegenerative diseases such as Alzheimer's, Parkinson's, Menkes, Wilson, familial amyotrophic lateral sclerosis, and prion diseases.[6,7] Therefore, the limit of copper in drinking water as set by the US Environmental Protection Agency (EPA) is 1.3 ppm (~20 μM).[8]

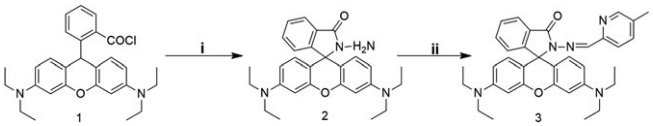
Designing heterocycle-based fluorescent sensors *that is*, rhodamine based,[9,10] benzimidazole based,[2,7] anthracene based,[11] biphenyl based,[12] dipyrromethene (BODIPY),[8] calix[4]arene based,[13,14] chromenone based,[5] cyclodextrin based,[15] indene-1,3-dione based,[16] phenylenediamine based,[17] thiazole based,[18] carbazole based,[19] ferrocene based,[20] and pyrrole based,[21] is an interesting strategy for probing a minute copper level in the sample. Fluorescent probes made up of organic materials for the heavy metal ion detection attracted widespread interest for environmental detection of contaminants. It has the advantageous features including high sensitivity, quick response, and nondestructive nature. There are several reports on the organic material-based fluorescent sensors for trace copper detection but only a few exhibited the applications of the proposed sensor toward imaging of live cells.[11,22–28]

Schiff bases are the important scaffold employed broadly in multiple dimensions for chemical production and scientific research as chelating agents, stabilizers, biologically active agents, analytic, and catalytic reagents.[29] In the present investigation, we have synthesized a 2-formyl-5-picolinyl-substituted rhodamine B derivative by treatment of rhodamine B hydrazide with 2-formyl-5-picoline and evaluated their complexation tendency toward several metal ions. Rhodamine B substituted with the picoline acid via amide linkage was reported by Liu et al.[30] for the copper ion detection by the reaction of rhodamine B acid chloride and pyridine-2-carboxylic acid hydrazide through the amide bond formation. The present Schiff base derivative reported possesses longer electronic dense cloud over the lower loop of the molecular skeleton, which is of benefit to bind efficiently with the copper ions. Furthermore, it is more facile to generate the Schiff base ligand via imine formation reaction under gentle experimental conditions. We have further employed our reported ligands for copper signaling inside the live cells and determined the toxicity profile of the ligand. We believe that the present findings will be helpful for the future designing of a metal ion detector via imine linkage, which is compatible for live cell imaging due to its membrane permeable efficacy. High product yield, ease of synthesis, and low toxicity of the probe are the features that inspire us to select the target compound.

2 | RESULTS AND DISCUSSION

2.1 | Synthesis of ligand 3

The rhodamine B acidic functionality was transformed into acid chloride by the treatment with phosphorous oxychloride, which was then treated with the hydrazine hydrate to afford the corresponding hydrazide. The treatment of 2-formyl-5-picoline with the freshly prepared rhodamine hydrazide affords the targeted Schiff base derivative. The disappearance of the broad acidic hydroxyl peak in the range of 3,400–2,500 cm^{-1} in the FT-IR spectrum indicates the conversion of rhodamine acid into its chloride. The rhodamine hydrazide formation was visually detected by the change of color of the material into off while solids, which affords a broad signal in the range of 3,389–3,340 cm^{-1} for primary amide stretching vibration. The targeted Schiff base formation was indicated by the FT-IR spectral investigation by the emergence of a signal at 1,586 cm^{-1}, which was assigned to the C=N stretching vibration. The synthetic pathway adopted to get the target molecule is shown in Scheme 1.



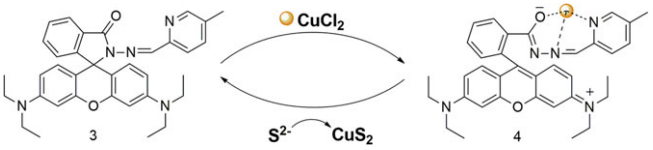
SCHEME 1 Synthesis of ligand **3**; reagents and conditions. (i) Hydrazine, methylene chloride, reflux; (ii) 2-formyl-5-picoline, methylene chloride, and reflux

2.2 | Ligand–metal chelation

The ligand–metal complexation mechanism was assessed using optical measurements as well as FT-IR spectroscopic analysis. The colorimetric change in the ligand solution after copper ion addition as well as the emergence of new absorption and emission signals represent the conformational changes in the ligand skeleton triggered by the copper ions. There was slight shifting of the amide carbonyl as well as C=N signal position after complexation with the copper ions indicating the attachment of the copper to the receptor ligation site. In the case of nuclear magnetic resonance (NMR) spectral analysis, there was no noticeable disappearance or shifting in the signals after copper complexation representing that the molecular skeleton remains the same involving only the electronic rearrangement. The reversibility of the receptor with that of the sulfide ions confirms the complexation process via colorimetric as well as florigenic variation. Colorimetric as well as chromogenic features of the ligand-copper complex were recovered upon induction of the sulfide ion, which was due to the formation of copper sulfide, as copper was displaced from its position, it will regenerate the entire ligand features (Scheme 2).

2.3 | Spectroscopic properties

The spectroscopic properties of the ligand were investigated in aqueous/acetonitrile (40:60, v/v) and the corresponding spectra are shown in Figure 1. The free ligand solution was colorless and nonemissive due to its existence in the



SCHEME 2 Proposed spirolactam ring-opening mechanism of ligand **3** upon copper chelation in acetonitrile/water (6:4, v/v) at neutral pH and its reversibility with sulfide ions

spirolactam conformation while upon copper ion addition there was a chromogenic response in the reaction solution from the colorless to the light pink. This chromogenic change was due to the conformation changes in the ligand molecule upon chelation with the metal ions. The UV–visible properties of the ligand were investigated upon treatment of the ligand solution with 0.5 μM copper ion and the other metallic species including Sc^{3+}, Yb^{3+}, In^{3+}, Ce^{3+}, Sm^{3+}, Cr^{3+}, Sn^{2+}, Pb^{2+}, Ni^{2+}, Co^{2+}, Ba^{2+}, Ca^{2+}, Mg^{2+}, Ag^{+}, Cs^{+}, Cu^{+}, and K^{+} in aqueous/acetonitrile (40:60, v/v, pH 7.0.) at ambient temperature. The ligand shows no response upon induction of Sc^{3+}, Yb^{3+}, In^{3+}, Ce^{3+}, Sm^{3+}, Cr^{3+}, Sn^{2+}, Pb^{2+}, Ba^{2+}, Ca^{2+}, Mg^{2+}, Ag^{+}, Cs^{+}, Cu^{+}, and K^{+} while less intense absorption spectrum was appeared in the case of Ni^{2+} and Co^{2+}. Interestingly, the high-intensity absorption band was observed with the maximum absorption at 558 nm upon copper ion induction, which was utilized for the sensing of the copper ions. The ratiometric response of the sensor toward the copper ions was determined by the successive induction of the copper ions from 0.2 to 1.4 equivalent into the 0.5 μM ligand solution. The results showed that there was constant increment in the absorption signal intensity at 558 nm upon successive copper ion induction and these results might be utilized for the ratiometric copper ion detection (Figure 1).

The similar response of the ligand toward the copper ions was observed upon fluorescent spectral measurement. The addition of the copper ion into the ligand solution triggered an intense emission signal at 591 nm whose intensity continues to increase upon successive copper ion addition from 0.2–1.4 equivalent. The fluorescent titration graph was also utilized to find out the limit of detection of 0.54×10^{-9} mol/L for the copper ions via 3 σ/slope. The slope was obtained utilizing the inset graph (Figure 2). From the titration graph, the association constant value was determined to be 1.19×10^{5} M^{-1}.

The quick response by the sensor toward the metal ion recognition is a prerequisite to obtain better results. To understand this, the fluorescent emission signal intensity was observed with different time intervals from 1 to 10 min. The results showed that the maximum emission signal intensity was observed around 1–2 min of the sample preparation and these results were quite satisfactory for a good sensing material. Above 2 min of incubation, the emission spectral results become stable as shown in Figure S1, Supporting Information.

The practical applicability of the sensor toward the copper ions was determined by utilizing the distilled water as well as tape water and the same emission response was observed by either of the solvent representing that proposed material can be utilized directly for the metallic contaminant assessment in the tape water, lake, or river water (Figure S2). Moreover, this experiment represents that there was no metallic contamination in our tap water used.

The effect of the pH was studied in the buffer solution (PBS and Tris HCL buffer) ranging from pH 2–8 as shown in Figure S3. The ligand exhibited the absorption and emission signals in the acidic pH range, which represent that the spirolactam ring of the synthesized probe was fragile and underwent ring opening involving the protonation pathway. In the neutral pH range, the ligand was supposed to exist in

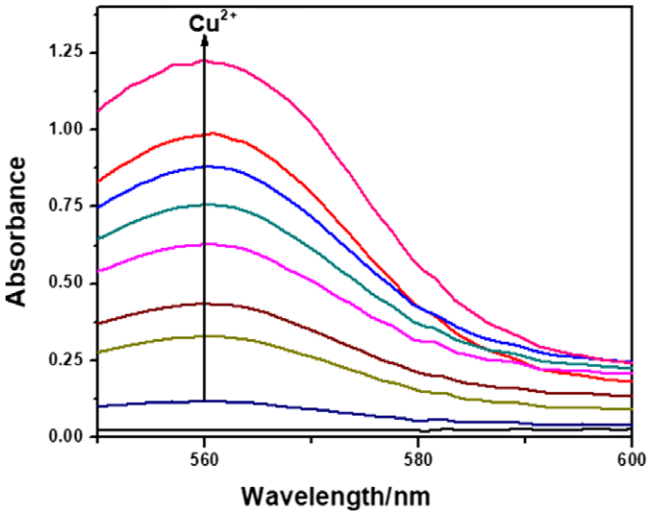


FIGURE 1 UV–visible absorption spectral titration of ligand **3** (0.5 μM) in the presence and absence of Cu^{2+} (0.2–1 eq) in acetonitrile/water (6:4, v/v) at pH = 7.0

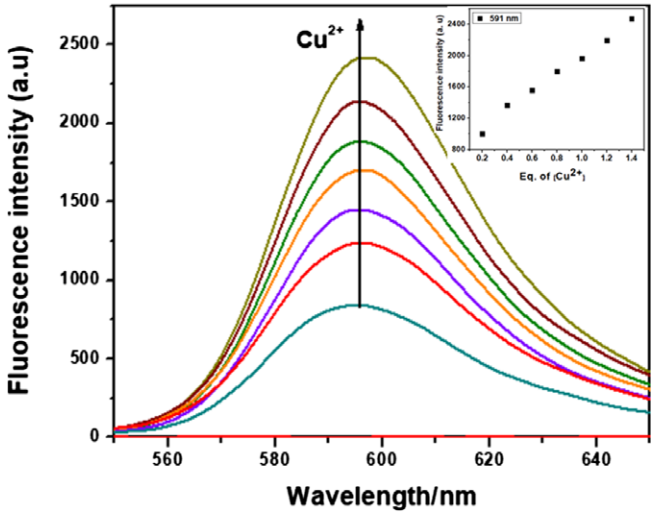


FIGURE 2 The fluorescence titration of ligand **3** (0.5 μM) at emission maxima of 591 nm as a function of copper concentration (0.2–1 eq), the inset describes the fluorescence enhancement at emission maxima of 591 nm

the spirolactam ring closure conformation. The copper ion + ligand solution was still emissive in the neutral pH range representing the conformational change in the ligand triggered by the copper ions. In the basic environment, there was again downfall in the emission signal intensity of the ligand copper complex because of decreasing the copper ion concentration due to involvement of the copper in the generation of $Cu(OH)_2$.

Fluorescence quantum yield of ligand-Cu^{2+} complex solution was calculated to be ΦFL = 0.53 (relative to the standard rhodamine B in acetonitrile, $\Phi_{std} = 0.59$), using Equation 2. The ligand showed 0.0071 values for the fluorescence quantum yield.[31–33]

$$\Phi_{unk} = \Phi_{std}\,(I_{unk}/A_{unk})(A_{std}/I_{std})(n_{unk}/n_{std}), \qquad (1)$$

where Φ_{unk} is the fluorescence quantum yield of the sample, Φ_{std} is the quantum yield of the standard, and I_{unk} and I_{std} are the integrated fluorescence intensities of the sample and the standard, respectively, A_{unk} and A_{std} are the absorbances of the sample and the standard at the absorption wavelength, respectively, nunk and nstd are the refractive indices of the corresponding solutions.

The effect of the solvent on the ligand-copper complexation process was established by utilizing different percentage of the organic and mixed aqueous organic solvents. The ligand becomes precipitated in the pure water while becomes soluble upon increasing the percentage of the organic solvent. The suitable ratio of the aqueous-organic solvent was determined by the series of experiments and the results are shown in Figure S4.

The experimental results including absorption signal intensity at the absorption maxima of 558 nm and molar absorptivity, obtained by varying solvents and their ratios, are tabulated in Table 1. The minimum absorption signal intensity was observed in the pure aqueous media due to the ligand precipitation while there was the same results for the mixed organic-aqueous solvents including acetonitrile: water, methanol: water, and ethanol: water.

3 | BIOIMAGING ANALYSIS

To trace the copper contamination in the intracellular media, the confocal fluorescent microscopic analysis was conducted utilizing BHK-21 (hamster kidney fibroblast) cells. The experimental results exhibited that there wasno deformation in the cells during the course of study showing the minimum

TABLE 1 The effect of solvents on the copper ligand complexation

S. No.	Solvents	Abs. Intensity at 558 nm
1	H_2O	0.019
2	MeCN:H_2O	1.24
3	MeOH:H_2O	1.24
4	EtOH:H_2O	1.24

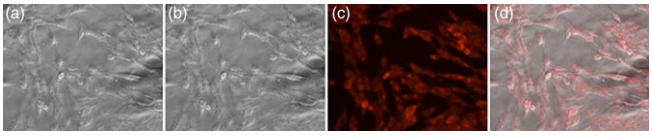


FIGURE 3 Confocal fluorescence microscopic images of BHK-21 cells; (a) BHK-21 cells without incubation with the probe (control); (b) bright filed images of BHK-21 cells incubated with probe (0.5 μM); (c) fluorescence images of BHK-21 cells incubated with probe (0.5 μM) in the presence of (1 eq) copper ion; and (d) merged images of BHK-21 cells incubated with probe (0.5 μM) in the presence of (1 eq) copper ion

level of probe toxicity toward the tested cell lines. Moreover, there was bright red fluorescence coming from the probe-incubated cells upon treatment with the copper ions. The increasing concentration of the copper ions brings more reddishness from the cells. This increment in the fluorescence brightness upon successive copper induction can be utilized to find out the metal contamination level inside the cells in the ratiometric manner (Figure 3).

3.1 | Cytotoxicity analysis

The toxicity analysis of the receptor was carried out by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] analysis. The assay was performed after 4 and 24 hr of ligand treatment with BHK-21 (hamster kidney fibroblast). The result represents 100% cell viability toward the tested cell lines (Figure 4).

4 | EXPERIMENTAL

4.1 | Substrate and reagents

Rhodamine B, 2-formyl-5-picolyl, hydrazine hydrate and hydrazine in THF, glacial acetic acid, phosphorous oxychloride, and $NaHCO_3$ were purchased from Aldrich. Ethanol, methanol, 1,2-dichloroethane, methylene chloride, acetonitrile, acetone, water, dimethyl sulfoxide, hexane, and

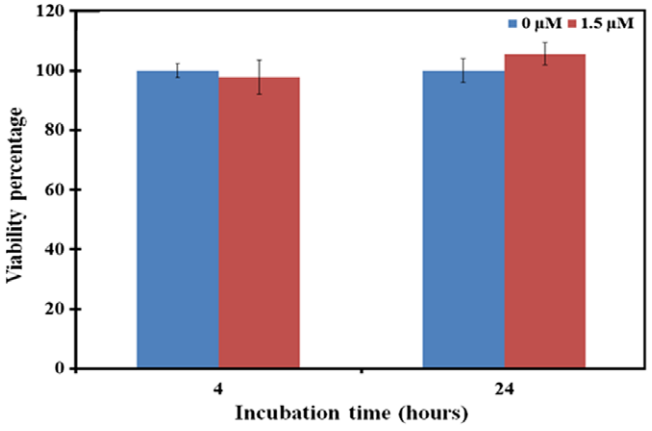


FIGURE 4 Cell viability of BHK-21 cells cultured in complete media with ligand **3** (0.5 μM) while the control cells were cultured in the medium without ligand

ethyl acetate (Samchun Chemicals, Korea), and $Sc(OTF)_3$, $YbCl_3{\cdot}6H_2O$, $InCl_3$, $CeCl_3$, $SmCl_3{\cdot}6H_2O$, $CrCl_3{\cdot}6H_2O$, $SnCl_2$, $PbCl_2$, $FeCl_3{\cdot}nH_2O$, $NiCl_2{\cdot}6H_2O$, $CoCl_2{\cdot}6H_2O$, $CuCl_2{\cdot}2H_2O$, $BaCl_2{\cdot}2H_2O$, $CaCl_2{\cdot}2H_2O$, CsCl, CuCl, and KCl (Aldrich and Alfa Aesar) were used during experiment. The major chemicals utilized for biological studies include minimum essential media (MEM, Wel Gene, Korea), fetal bovine serum (FBS, Bio West USA), Tripsin (Thermo Scientific, South Loga, UT), PBS (Wel Gene), and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma-Aldrich, USA].

4.2 | Instrumentations

The reaction progress was monitored by thin layer chromatographic (TLC) analysis, and the R_f values were determined by employing precoated silica gel aluminum plates, Kieselgel $60F_{254}$, from Merck (Germany), using dichloromethane: methanol, 9:1, as an eluent. TLC was visualized under a UV lamp (VL-4. LC, France). The FT-IR spectra were recorded in KBr pellets on a SHIMADZU FTIR-8400S spectrometer (Kyoto, Japan). Proton and carbon nuclear magnetic resonance (^{1}H NMR &^{13}C NMR) spectra were recorded on a Bruker Avance 500 MHz spectrometer with TMS as an internal standard. The chemical shifts are reported as δ values (ppm) downfield from the internal tetramethylsilane of the indicated organic solution. Peak multiplicities are expressed as follows: s, singlet, bs, broad signal, d, doublet, t, triplet, q, quartet, and m, multiplet. The coupling constants (J values) are given in hertz (Hz). Mass spectra were recorded on the AB SCIEX Co. 4000 QTRAP LC/MS/MS System. Abbreviations are used as follows: DMSO-d_6, Dimethyl sulfoxide-d_6; FT–IR spectroscopy, Fourier transform infrared spectroscopy, MC, Methylene chloride, and DMAP, 4-Dimethylaminopyridine.

4.3 | General procedure for the synthesis of ligand 3

Rhodamine B Schiff base ligand **3** was synthesized from the rhodamine B hydrazide **2** (1 eq) in the presence of 2-formyl-5-picolyl (1 eq) and a catalytic amount of glacial acetic acid. The rhodamine B hydrazide **2** was synthesized upon treatment of rhodamine B acid chloride with hydrazine under reflux conditions. The rhodamine B acid chloride was prepared by the reaction of rhodamine B and phosphorus oxychloride in the presence of methylene chloride as a solvent. The final product was purified by column chromatography and utilized for the analysis.

4.3.1 | 3′,6′-Bis(diethylamino)-2-(([5-methylpyridin-2-yl]methylene)amino)spiro[isoindoline-1,9′-xanthen]-3-one (3)

Light yellow powder; yield: 78%; ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.55 (aromatic, 1H, s), 7.81–7.78 (aromatic, 3H, m), 7.67 (aromatic, 1H, t, J = 7 Hz), 7.55–7.46 (aromatic, 2H, m), 7.00–6.98 (aromatic, 1H, m), 6.31(aliphatic imine, 1H, s), 6.43–6.31 (aromatic, 5H, m), 3.35–3.29 (aliphatic, 8H, q, J = 10 Hz), 2.84–2.80 (aliphatic, 1H, q, J = 8.5 Hz), 1.28 (NH, 2H, bs), 1.09–1.07 (aliphatic, 12H, t, J = 9 Hz), and 2.28 (aliphatic, 3H, s); ^{13}C NMR (125 MHz, DMSO-d_6) δ175.9, 152.3, 152.2, 151.8, 149.7, 149.3, 148.3, 135.6, 136.1, 129.9, 128.8, 128.4, 127.8, 126.9, 117.3, 116.1, 48.1, 17.7, and 12.8; MS for $C_{35}H_{37}N_5O_2$(ESI, m/z), 560.6 $[M + H]^+$. Anal. Calcd. For $C_{35}H_{37}N_5O_2$: C, 75.07; H, 6.54; and N, 12.31; Found: C, 75.10; H, 6.62; and N, 12.49%.

4.4 | General procedure for spectroscopic assay

Stock solution of the ligand (300 μM) was prepared by dissolving 1.679 mg ligand in methanol (total volume 10 mL). Similarly, to prepare Cu^{2+} stock solution (300 μM), 1.344 mg of copper (II) chloride was dissolved in distilled water (total volume 10 mL) to get 1 mmol solution, then 3 mL of this solution was diluted to 10 mL with distilled water to get 300 μM Cu^{2+} stock solution. All the metal ion solution was prepared using the procedure similar to that used for preparation of Cu^{2+} stock solution. For spectroscopic measurements, test solution of 1 mL was prepared with 780 μL of 40% aqueous methanol, 10 μL of ligand stock solution, 0.1 mL of buffer solution (10 mM), and 10 μL of Cu^{2+} stock solution. The resulting solution was mixed before measurement and the final volume was fixed as 1 mL for UV–visible and Fluorescent measurement using a [SCINCO] UV–Vis Spectrophotometer "S-3100" and a FS-2 fluorescence spectrometer (SCINCO, Korea), respectively.

4.5 | General procedure for the MTT assay

The MTT assay was carried out following the reported procedure.[34] Briefly, the cells were incubated with Cu^{2+}(50 μM) and ligand **3** (60 μM) for 24 hr. Then, cells were washed with phosphate-buffered saline (PBS), and incubated with *Dulbecco's Modified Eagle's medium* (DMEM medium, 200 μL/well) containing 50 μL of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 5 mg/mL] solution. Following 2 hr incubation at 37°C, growth medium was removed gently from the plate and 200 μL/well dimethyl sulfoxide was added to solubilize the produced purple formazan crystals. Later, the absorbance for each well was measured at 560 nm using microplate spectrophotometer systems (BioTek, synergy HT) and the results were calculated in percentage with respect to untreated sample called control.

5 | CONCLUSIONS

In summary, a simple, economic, and efficient rhodamine B Schiff base derivative 3′,6′-Bis(diethylamino)-2-(([5-methylpyridin-2-yl]methylene)amino)spiro[isoindoline-1,9′-xanthen]-3-one (**3**) was synthesized by treatment

of rhodamine B hydrazide with 2-formyl-5-picoline. The synthesized material was characterized by the optical, FT-IR, NMR, and mass spectrometric analysis and evaluated its potential as a metal ion sensor upon treatment with varieties of inorganic salts. The results exhibited a noticeable chromogenic and fluorogenic response of the ligand solution upon treatment with copper ions indicating the ligand-copper complexation. The complexation process was found to turn down upon addition of sulfide ions into the complex solution due to the formation of copper sulfide. To evaluate the utility of the reported probe for copper detection inside the cellular media, the bioimaging experiment was performed using BHK-21 (hamster kidney fibroblast) cells and studied under a confocal fluorescence microscope. The appearance of bright red fluorescence from the ligand-mixed cells upon treatment with copper ions without any cell deformation reflects excellent membrane permeability and the minimum level of toxicity of the reported sensor. The cytotoxic analysis of the receptor was further carried out using the MTT assay, and satisfactory results were obtained.

CONFLICTS OF INTEREST

The authors declare no potential conflict of interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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