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ORIGINAL ARTICLE

Rhodamine Diaminomaleonitrile Conjugate as a Novel Colorimetric Fluorescent Sensor for Recognition of Cd^{2+} Ion

Perumal Sakthivel[1] · **Karuppannan Sekar**[1] · **Gandhi Sivaraman**[2] · **Subramanian Singaravadivel**[3]

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Abstract Rhodamine diaminomaleonitrile linked probe (**RD-1**) shows highly sensitive colorimetric and selective turn-on fluorescent response to Cd^{2+} over other metal ions. The fluorescence intensity and absorbance of the probe **RD-1** showed a good linearity, with very low detection limits of 18.5 nm. The probe **RD-1** was preliminarily applied to the determination of Cd^{2+} ion in water samples from river and tap water with satisfying results. The live cell image confocal microscopy, HeLa cell demonstrated that **RD-1** had low cytotoxicity with good membrane permeable property is successfully applied to fluorescence microscopic imaging for the detection of Cd^{2+} ions.

Keywords Rhodamine · Diaminomaleonitrile · Cadmium · Sensor · Fluorescence

Highlights
- Rhodamine diaminomaleonitrile based probe for colorimetric and fluorescent detection of Cd(II) was developed.
- The detection limit of sensor is 18.5 nm.
- The probe can be used to detect Cd(II) in live cells.

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✉ Karuppannan Sekar
karuppannansekar@gmail.com

✉ Subramanian Singaravadivel
vesp1984@gmail.com

[1] Department of Chemistry, Anna University - University College of Engineering, Dindigul 624622, India

[2] Institute for Stem Cell Biology and Regenerative Medicine, Bangalore 560065, India

[3] Department of Chemistry, SSM Institute of Engineering and Technology, Dindigul 624002, India

Introduction

The development of luminescent chemical devices is an active field of research in supramolecular chemistry [1–3]. An important area within this field is the development of luminescent chemosensors [4–8]. Such sensors have the advantage of possessing high sensitivity and selectivity, as well as providing on-line and real-time analysis that has revolutionized the field of chemical analysis, particularly in critical care analysis of blood and serum samples [9, 10].

Cadmium, an essential element in the earth, is widely used in agriculture and industry. These sources of cadmium cause excessive exposure to human society. Whereas Cd^{2+} is also known for its toxicity, which can induce serious health and environmental problem, such as renal dysfunction, Calcium metabolism disorders, prostate cancer, etc. [11, 12]. The EPA (United States Environmental Protection Agency) gives an enforceable drinking water standard for Cd^{2+} of 5 ppb to prevent kidney damage and other related diseases, while the WHO (World Health Organization) provides a more strict guideline value for Cd^{2+} of 3 ppb for drinking water [13].

Fluorescence is a powerful method to detect ions and neutral molecules owing to its operational simplicity and high sensitivity. The challenge in development of any fluorescent sensor is the induced signal change when a target specifically binds to the probe [14–17].

Several sensors have been developed and utilized for Cd^{2+} imaging in living cells [18, 19]. In spite of their fascinating responses to Cd^{2+}, these sensors still have some hang-ups, such as simple response, UV excitation, and large spectral overlap [20, 21]. To date, it is still a tremendous challenge to design Cd^{2+} selective sensors for the accurate detection of Cd^{2+} in aqueous solutions and biological environments [22, 23].

The well-known fluorophore rhodamine exists in two forms; one is the spirolactam and the other is the amide form.

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The spirolactam form does not exhibit absorption and emission, whereas the amide form exhibits absorption and emission in the visible region. When metal ions or analyte binds with the rhodamine dye, the spirolactam ring can be readily converted into the amide form. This has been utilized for the development of chemosensors for metal ions [24–34].

Diaminomaleonitrile represents a class of π conjugated compound with electronic donor and acceptor parts with high electron affinity nature and interesting binding properties [35].

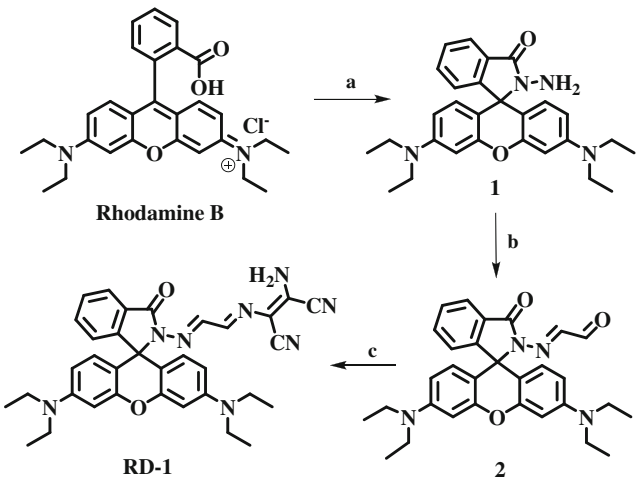
There are very few reports on cadmium sensing based on rhodamine dyes through spirocyclic ring opening mechanism [36–38]. In this paper, we developed a rhodamine based probe **RD-1** linked to diaminomaleonitrile moiety (Scheme 1). This could be used for the detection of Cd^{2+} with high sensitivity and selectivity, good linearity, and showed significant colorimetric and fluorometric response in a short time. The low cytotoxicity and cell-membrane penetrability of **RD-1** were confirmed by MTT assay and cell imaging, respectively.

Experimental

Materials and General Methods

All reagents and solvents were used without purification. Rhodamine B and diaminomaleonitrile were purchased from Sigma Aldrich. Metal chloride salts procured from Merck were used as the source for metal ions.

Absorption measurements were carried out using an Antech (AN-UV-7000) UV-vis spectrophotometer. Fluorescence spectra were recorded on F-4500 Hitachi fluorescence spectrophotometer. The slit width was 5 nm for both excitation and emission. NMR spectra were recorded on a Bruker (Avance) 300 MHz instrument using TMS as internal standard. ESI-MS spectral analysis was performed in positive ion mode on a liquid chromatography-ion trap mass spectrometer (LCQ Fleet, Thermo Fisher Instruments Limited, US). Fluorescence microscopic images were taken with a Nikon fluorescence microscope using a filter.



Scheme 1 Synthesis of Probe **RD-1** **a** Hydrazine-hydrate, MeOH, reflux, 65 °C, 12 h. **b** Glyoxal, EtOH, rt. **c** 2,3-diaminomaleonitrile, EtOH, 70 °C, 6 h

Synthesis

Synthesis of 2-Amino-3",6"-Bis(Diethylamino) Spiro[Isoindoline-1,9"-Xanthen]-3-One **1***:*

Rhodamine B hydrazide was synthesized for the following reported procedure [39]. Rhodamine B 4 g (6.26 mmol) was dissolved in 40 ml of methanol and added excess amount of hydrazine hydrate drop wise to the solution. The reaction mixture was refluxed until red colour disappeared. After completion of reaction, the reaction mixture was cooling to room temperature and the solution was poured into 400 ml of distilled water for 6 h. Then the solid precipitate was filtered and dried in vacuum to give compound **1** in pale pink solid. Yield: (88%).

Synthesis of (E)-2-((3",6"-Bis(Diethylamino) -3-Oxospiro[Isoindoline-1,9"-Xanthen]-2-Yl)Imino) Acetaldehyde **2***:*

The compound **1** (700 mg, 1.54 mmol) was dissolved in 40 ml of ethanol, an excess amount of 40% aqueous glyoxal was added drop wise to the reaction mixture and stirred at room temperature for 12 h. After the reaction is completed 50 ml of saturated sodium chloride solution was added to obtain the pale yellow precipitate. The precipitate was filtered and dried in vacuum and purified by column chromatography in hexane/ethyl acetate mixture (9:1) afforded 0.38 g of compound **2** in 68% yield.

^{1}H NMR (300 MHz, $CDCl_3$), δ (ppm): 9.38–9.36 (d, 1H, J = 7.5 Hz), 7.98–7.96 (d, 1H, J = 7.2 Hz), 7.48–7.41 (m, 4H), 7.27 (d, 1H, J = 7.5 Hz), 7.05 (d, 1H, J = 6.83 Hz), 6.32–6.40 (m, 2H), 6.15–6.19 (m, 2H), 3.21 (q, 8H, J = 6.9 Hz), 1.07 (t, 12H, J = 6.9 Hz).

Synthesis of (2-Amino-3-(((1E,2E) -2-((3",6"-Bis(Diethylamino) -3-Oxospiro[Isoindoline-1,9"-Xanthen]-2-Yl)Imino) Ethylidene)Amino)Maleonitrile) **RD-1***:*

The compound **2** (500 mg, 1.01 mmol) was dissolved in 30 ml of ethanol and diamino maleonitrile (1.01 mmol) was added to the solution and refluxed 70 °C for 6 h. After completion of reaction the precipitate was filtered and washed with ethanol. The crude product was purified by column chromatography with hexane/ethyl acetate (8/2, *v*/v), brown solid was obtained (0.38 g, 65%).

FT-IR (KBr, cm^{-1}): 699, 787, 1115, 1222, 1262, 1307, 1374, 1513, 1591, 1613, 1699, 2205 and 2967.

^1H NMR (300 MHz, $CDCl_3$), δ (ppm): 8.41–8.38 (d, 1H, $J = 7.8$ Hz), 8.01–7.97 (m, 2H), 7.54–7.46 (m, 3H, ArH), 7.13–7.10 (d, 1H, $J = 7.8$ Hz), 6.49–6.43(m, 3H), 6.28–6.25 (m, 2H), 5.29 (s, 2H), 3.30 (q, 8H, $J = 6.6$ Hz), 1.15 (t, 12H, $J = 6.9$ Hz).

ESI-Ms.: m/z $[M + H]^+$: 586.28; found: 586.58.

Stock Solution Preparation for Spectral Detection

The chloride or nitrate salts of Ag^+, Al^{3+}, Ba^{2+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Cr^{3+}, Cu^{2+}, Fe^{3+}, Hg^{2+}, K^+, Mg^{2+}, Mn^{2+}, Na^+, Ni^{2+}, Pb^{2+} and Zn^{2+} were prepared in acetonitrile-water (7:3) mixture as stock solutions (1 mmol). The **RD-1** stock solution (1 mmol) was prepared in acetonitrile-water (7:3) mixture. The working solutions of **RD-1** were freshly prepared by diluting the highly concentrated stock solution to the desired concentration prior to spectroscopic measurements.

UV-Vis and Fluorescence Titration Studies

The absorption and fluorescence responses of the probe **RD-1** towards various metal ions was investigated by UV-vis spectroscopy and fluorescence spectroscopy respectively in acetonitrile-water (7:3) mixture.

MTT Assay

The cell viability of the probe **RD-1** were tested against HeLa cell lines using the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The cells were seeded into a well plate at a density of 1.5×10^4 cells per well and incubated in medium containing **RD-1** at concentrations ranging from 0 to 50 μM for 30 min. To each well, 100 μL of MTT was added and the plates were incubated at 37 °C for 1 h to allow MTT to form formazan crystals by reacting with metabolically active cells. The medium with MTT was removed from the wells. Intracellular formazan crystals were dissolved by adding 100 μL of DMSO to each well and the plates were shaken for 10 min. The absorbance was recorded using Plate reader.

Cell Culture and Fluorescence Imaging

HeLa cells were grown in modified Eagle"s medium supplemented with 10% FBS (fetal bovine serum) at 37 °C. The HeLa cells were incubated with the probe **RD-1** (10 μM in DMSO/H_2O (7:3, *v*/v) buffered with HEPES buffer) and imaged through fluorescence microscope. The cells were washed with HEPES three times to remove the excess of the probe **RD-1** in the extra cellular parts and growth medium. Again the probe treated cells were further incubated with $Cd(NO_3)_2$ (10 μM) for 10 min at 37 °C and imaged with Nikon fluorescence microscope.

Results and Discussion

Synthesis of RD-1

Compound **2** (1 mmol) and diaminomaleonitrile (1 mmol) were mixed with 20 mL ethanol and the mixture was stirred under reflux condition for 6 h. The suspension was filtered, and then the solid was dried in vacuum. The residue was purified by column chromatography hexane/ethyl acetate (8/2, *v*/v) as elutent to afford brown solid in a yield of 65%. **RD-1** was characterized by FT-IR, NMR and Mass spectral analysis (Fig. S1-S3).

The absorption spectra of probe **RD-1** (1:1) toward various metal ions such as Na^+, K^+, Ca^{2+}, Cu^{2+}, Cd^{2+}, Zn^{2+}, Cd^{2+}, Hg^{2+}, Pb^{2+}, Fe^{2+}, Cr^{3+}, Ni^{2+}, Al^{3+}, Ag^+ and Co^{2+} were investigated (Fig. 1). Upon addition of Cd^{2+}, an absorption peak emerged at 530 nm. The development of the noticeable naked-eye detection of the magenta colour in the probe upon addition of Cd^{2+} involves a metal-induced delactonization of rhodamine. Upon complexation, colourless spirolactam form is converted into its (colorless to pink) colored ring opened amide form. The change in the color and absorbance wavelength makes it feasible to distinguish Cd^{2+} from other metal ions. Upon addition of Cd^{2+} ion the probe **RD-1** leads to a linear relationship of gradual enhancement of the intensity at 530 nm (Fig. S4).

Fluorescence Titration and Selectivity

Changes of fluorescence emission spectra of the probe **RD-1** in the presence of various cations were studied in HEPES buffer (10 μM, pH 7.54). Fluorescence titration was used to further investigate the binding capability of **RD-1** for Cd^{2+}. The probe **RD-1** weak fluorescence upon excitation at 530 nm

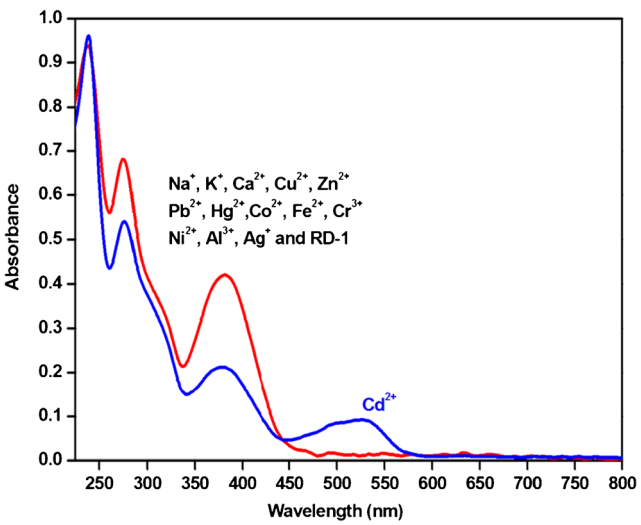


Fig. 1 UV-vis absorption spectra of probe **RD-1** (10 μM) upon addition of various metal ions in 10 μM HEPES buffer solution

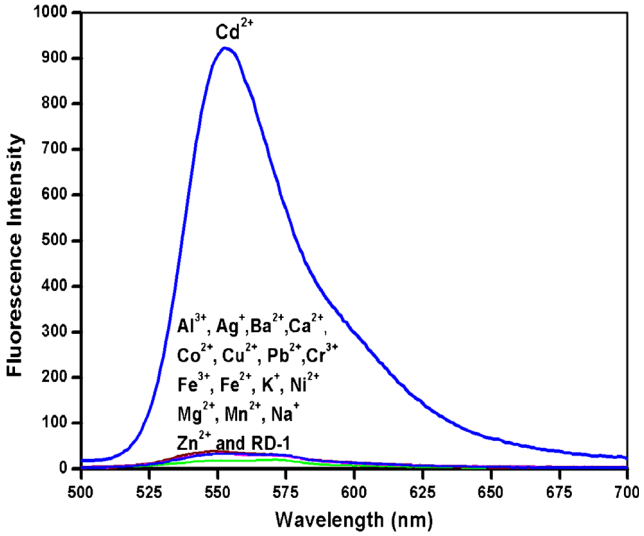


Fig. 2 Fluorescence spectra of probe **RD-1** (10 μM) upon addition of various metal ions in 10 μM HEPES buffer, λex = 530 nm

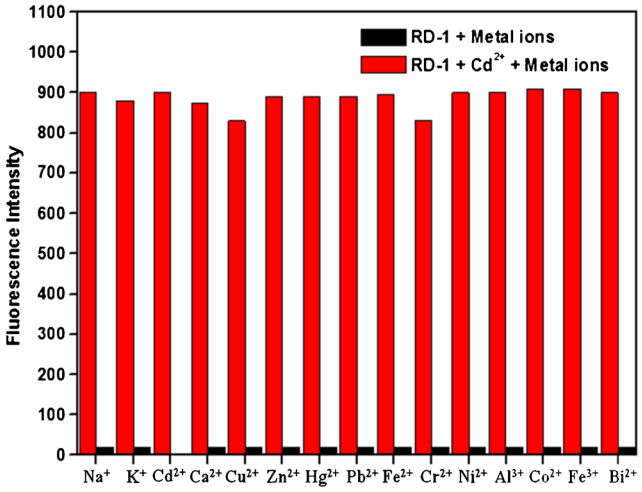


Fig. 4 Fluorescence response of 10 μM **RD-1** to various metal ions. Black bars represent the addition of metal ion to **RD-1**. Red bars represent the change of the emission upon the subsequent addition of Cd^{2+} to the above solution

because of its spirocyclic structure did not open upon the free **RD-1** (Fig. 2). Upon gradual addition of Cd^{2+} develops a significantly strong emission band at 553 nm (Fig. 3).

The addition of Cd^{2+} (0–20 μM) increased the fluorescence intensity by approximately 200-fold. The fluorescence intensity remained constant following the addition of greater than 50 μM (excess) Cd^{2+}. The enhancement in fluorescence intensity was likely due to the Cd^{2+}-triggered rhodamine spiroring opening reaction [40].

These results show that the probe **RD-1** is very selective and sensitive towards Cd^{2+} over other metal ions (Fig. 4). Job"s Plot analysis was used to find the stoichiometry for **RD-1**/Cd^{2+} complex. Job"s Plots exhibited the same maximum absorbance when the molar fraction of 0.5, indicating a 1:1 stoichiometry. ESI mass spectrometric analysis provided further support for the formation of the 1:1 complexes **RD-1**+ Cd^{2+}. A signal at 701.1776 corresponded to **RD-1**+ Cd^{2+}+H^+ were observed when Cd^{2+} was added to **RD-1** in methanol (Fig. S5 and S6).

On the basis of the 1:1 stoichiometry and UV–Vis titration data, the association constants (K_a) of the probe **RD-1** with Cd^{2+} were calculated using the Benesi–Hildebrand eq. [41] the measured fluorescence intensity [$1/(F - F_0)$] at 553 nm showed a linear relationship with $1/[Cd^{2+}]$, where F_0 and F are respectively the fluorescence intensities of the blank and sample solution containing **RD-1** and Cd^{2+}, and $[Cd^{2+}]$ are the concentrations of Cd^{2+} in the sample solutions. The association constant between **RD-1** and Cd^{2+} was 2.33 × 10^5 M^{-1}

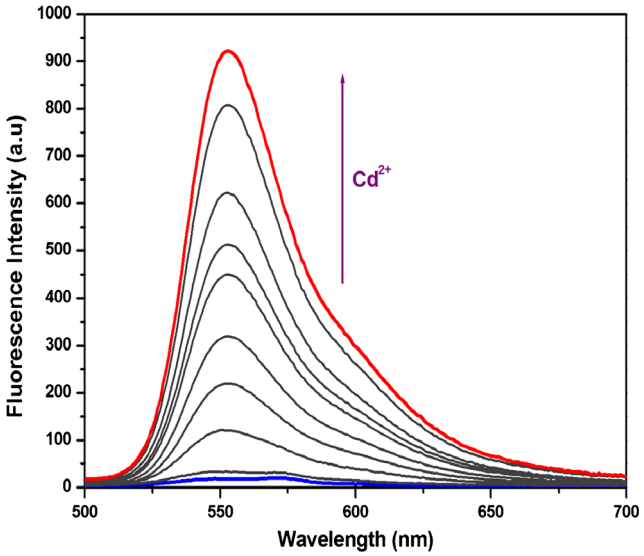


Fig. 3 Concentration-dependent fluorescence enhancement of **RD-1** (10 μM) on the addition of various amounts of Cd^{2+} (0–1.5 equiv) in 10 μM HEPES buffer solution (pH 7.54), λex = 530 nm

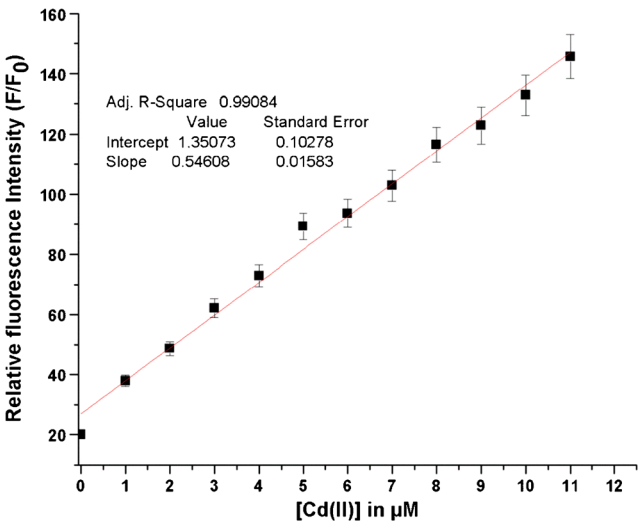


Fig. 5 Plot of change of fluorescence intensity at λ_{Emm} = 553 Vs concentration of Cadmium ions added to the probe **RD-1**

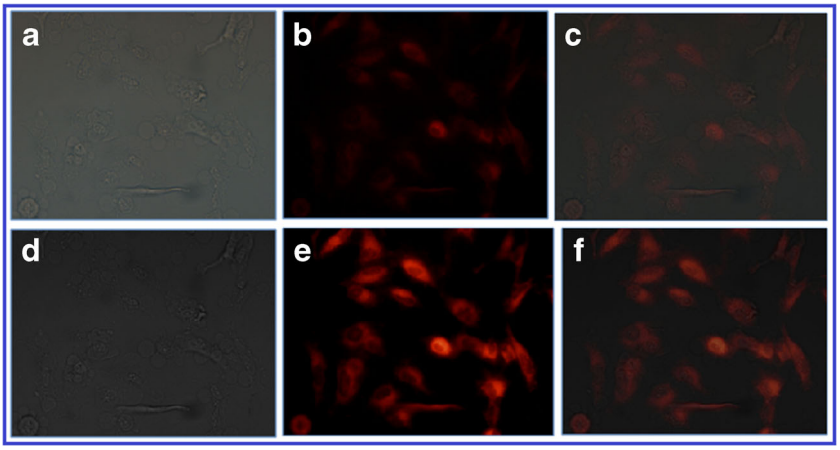


Fig. 6 **a** Brightfield images of HeLa cells incubated with **RD-1** (10 μM) for 30 min at 37 °C. **b** Fluorescence microscopic images of HeLa cells incubated with **RD-1** (10 μM). **c** Merged images of **a** and **b**. **d** Brightfield images **RD-1** pretreated HeLa cells and again treated with 10 μM $Cd(NO_3)_2$ for 10 min. **e** Fluorescence microscopic images HeLa cells incubated with Probe **RD-1** and 10 μM $Cd(NO_3)_2$ for 10 min. **f** Merged images of **d** and **e**

determined from fluorometric titration data [42]. Also, the detection limit of Cd^{2+} was determined from the absorbance titration profile as 18.5 nm based on 3σ/slope. The perfect linearity relationship between fluorescence intensity and the concentration of Cd^{2+} in the range of 1.0 * 10^{-7} to 1.0 *10^{-5} mol L^{-1} with a correlation coefficient of R^2 = 0.9908 was observed (Fig. 5). The LOD for Cd^{2+} detection by the probe **RD-1** is sufficient to detect Cadmium ions in living cells and biological systems.

The pH changes were investigated after the addition of different amounts of Cd^{2+} ion results indicated that the fluorescence enhancement is due to the coordination of Cd^{2+}, which induced the formation of a strongly fluorescent ring opened **RD-1**-Cd^{2+} complex. The pH study clearly elaborates the applicability of the probe for sensing Cd^{2+} in the physiological pH range (Fig. S7).

To further explore the complexation mode, FT-IR spectrometry was used to investigate the free ligand **RD-1** and their Cd^{2+}- complex (Fig. S8). We observed a shift of the stretching vibration frequency of the C = N bands from 1613 cm^{-1} and 1513 cm^{-1} (free **RD-1**), to slightly changes **RD-1**+ Cd^{2+} complex the C = N bands 1591 cm^{-1} and 1374 cm^{-1}, the stretch vibration of the carbonyl group of **RD-1** is 1699 cm^{-1} almost disappeared in the (**RD-1**+ Cd^{2+}) complex, these data indicate that the carbonyl and imines bond take part in coordination with Cd^{2+} complex.

Application in Living Cells

We next evaluated **RD-1** with HeLa cells to investigate the potential biological application of **RD-1** for fluorescence imaging. The cytotoxicity was evaluated by standard MTT

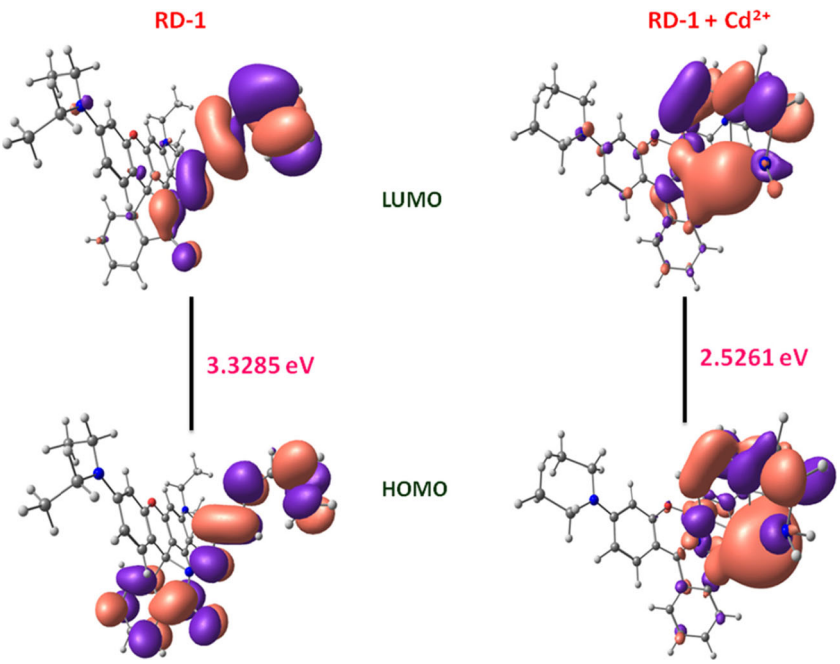


Fig. 7 Frontier molecular orbitals of **RD-1** and **RD-1** + **Cd^{2+}** obtained from the DFT calculations using Gaussian 09 program

Table 1 Quantification of Cd^{2+} in water samples with **RD-1**

Sample	Cd^{2+} added (μgL^{-1})	Cd^{2+} found (μgL^{-1})	Recovery (%)
Drinking water			
A	0	-	-
B	50	$50.01^{a} \pm 0.02^{b}$	100.02
C	100	$100.03^{a} \pm 0.03^{b}$	99.97
Tap water			
A	0	-	-
B	50	$50.04^{a} \pm 0.03^{b}$	99.89
C	100	$100.00^{a} \pm 0.06^{b}$	99.56
River water			
A	0	-	-
B	50	$50.06^{a} \pm 0.05^{b}$	99.78
C	100	$100.04^{a} \pm 0.06^{b}$	99.8

[a] Average of 3 measurements

[b] Standard deviation

(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The results indicate that the **RD-1** was non-toxic to the cells under the experimental conditions. The cells were incubated with a 10 μM solution of **RD-1** for 30 min at 37 °C in growth medium, and a very weak fluorescence was observed (Fig. 6b). In succession, the cells were added with $Cd(NO_3)_2$ (10 μM) for 10 min at 37 °C. After the cells were washed with 3 × 1 mL of PBS three times, an obvious fluorescence response from the intracellular region was observed. The bright field image confirmed that the cells were viable throughout the imaging experiments and the probe **RD-1** had good cell-membrane permeability, which could be used for detecting intracellular Cd^{2+} (Fig. 6, Fig. S9 and Fig. S10).

Density Functional Theory Studies

To get an insight into the electronic structure and the photo physical properties of **RD-1** and **RD-1**+ Cd^{2+} adduct, density functional theory (DFT) calculations was carried out with the Gaussian 09 program [43] with the B3LYP/6-311G and LANL2DZ for cadmium ions respectively. From the optimized geometries the DFT calculations were carried out using above mentioned sets. Frontier molecular orbital's derived from the optimized geometries, the HOMO and LUMO of **RD-1** is localized over the whole xanthenyl ring and diaminomaleonitrile unit, respectively, whereas in **RD-1** + Cd^{2+} the xanthenyl unit has HOMO character and the diaminomaleonitrile unit with reasonable contribution from Cd^{2+} has LUMO character (Fig. 7 and Fig. S11).

Practical Application

The potential utilities of **RD-1** for Cd^{2+} analysis in environmental water were performed. On the addition of water sample to the probe **RD-1** (10 μM), there is no change in the emission intensity whereas Cd^{2+} salts treated water samples showed a good linear dependences of fluorescence intensity enhancement. This result indicated that **RD-1** could detect Cd^{2+} in solutions thereby confirming its potential application for Cd^{2+} analysis in water. The recovery and RSD values for the addition of different concentrations of Cd^{2+} ions in water samples are given in Table 1.

Conclusions

In conclusion, herein we report the synthesis of a new probe **RD-1** based on rhodamine dye, conjugated with diaminomaleonitrile moiety, the colorimetric and fluorescent recognition of Cd^{2+} by **RD-1** were free from the interference of other metal ions. The chemosensor worked in aqueous solution at physiological pH ions with a very low detection limit of 18.5 nm. Further the non-toxicity of the probe makes it useful as an imaging agent for the detection of Cd^{2+} in living cells under physiological conditions.

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