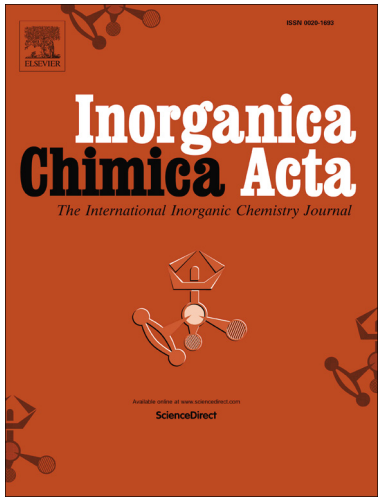

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Graphical Abstract

Single crystal X-ray structurally characterized palladium(II) selective fluorescence and colorimetric indicator for human breast cancer cell imaging

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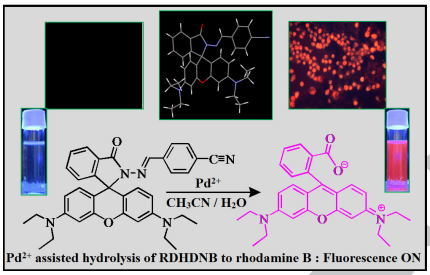

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Single crystal X-ray structurally characterized palladium(II) selective fluorescence and colorimetric indicator for human breast cancer cell imaging

Abhijit Ghosh[a], Sandip Nandi[a], Archya Sengupta[b], Ansuman Chattopadhyay[b], Sisir Lohar*[a] and Debasis Das[a*]

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ABSTRACT

Condensation of rhodamine B hydrazide with 4-formylbenzonitrile generates a Pd^{2+} selective probe (RDHDNB) that allows its naked eye and fluorescence recognition. Moreover, RDHDNB is useful to detect Pd^{2+} in human breast cancer cells MCF7, under normal and fluorescence microscope.

1. Introduction

Platinum group metals, specially palladium and its compounds, are widely used as catalysts in numerous chemical transformations applied for the synthesis of natural products, fine chemicals, therapeutic drugs, and polymers.[1-4] Innumerable organic reactions including Buchwald–Hartwig, Heck, Sonogashira, and Suzuki–Miyaura reactions are usually palladium catalyzed and have significant role in medicinal chemistry.[5-7] However, despite frequent and fruitful use of such reactions, resulting high level residual palladium in the product, even after purification, may lead to health hazard.[8] Additionally, frequent release of large quantity palladium to the environment occurs due to its extensive use in numerous commercial applications such as automobile catalytic converters, jewellery, dental alloys, fuel cells, medical instruments and electronics. Moreover, Pd^{2+} can bind thiol-containing amino acids, proteins (casein, silk fibroin, and many enzymes), DNA, macromolecules and bio-chemicals (vitamin B6), possibly impairing various cellular processes. Hence, palladium in environment can cause serious health problems like memory loss, dizziness, migraine headaches, allergies, immune system impairment and facial paralysis.[9]

So, trace level selective detection and estimation of Pd^{2+} is highly demanding. Traditional analytical methods for Pd^{2+} determination include atomic absorption spectrometry,[10] inductively coupled plasma atomic emission spectrometry,[11] inductively coupled plasma mass spectrometry,[12] solid-phase microextraction,[13] X-ray fluorescence[14] and capillary electrophoresis.[15] However, these methods suffer from complicated, time consuming sample preparation techniques or require sophisticated expensive equipment along with suitably trained analysts. Fluorescence sensing protocol is desirable for its easier and cost-effective methodology, good sensitivity and selectivity with high throughput fashion.[16] Most of the reported Pd^{2+} sensors are based on fluorescence quenching[17] while a few are "off–on" type.[18] Among the limited fluorescence "on" receptors reported in the literature reaction based chemodosimeter enabling Pd^{2+} promoted hydrolytic cleavage of specific bonds as sensing mechanism are rare.

Herein, single crystal X-ray structurally characterized, new rhodamine B hydrazide derived probe, RDHDNB (Scheme 1) can detect trace level Pd^{2+} both naked-eye and under UV light through recovery of rhodamine B by hydrolysis. Factors insist to select rhodamine as fluorophore include excellent photo-stability, impressive extinction coefficient and quantum yield with longer emission wavelength, favoring easy visualization. In presence of Pd^{2+}, colorless RDHDNB turns pink with significant fluorescence enhancement. To compare the Pd^{2+} selectivity of this rhodamine-based probe, RDHDNB, respective fluorescein compound (FLHDNB) have been synthesized as a model as shown in Scheme 2.

2. Experimental

2.1. Apparatus and reagents

A Shimadzu Multi Spec 1501 spectrophotometer is used for recording UV-Vis spectra. FTIR spectra are recorded on a PerkinElmer FTIR (model RX1) spectrophotometer. Mass spectra have been performed using a QTOF 60 Micro YA

263 mass spectrometer in ES positive mode. ^{1}H NMR spectra of RDHDNB and FLHDNB are recorded using Bruker Avance DPX 600 (600 MHz) in $CDCl_3$ and DPX 300 (300 MHz) in DMSO-d_6 respectively. The steady state fluorescence emission and excitation spectra are recorded with a Hitachi F-4500 spectrofluorimeter. Systronics digital pH meter (model 335) is used for pH measurement of the solutions. Dilute HCl or NaOH (50 μM) are used for pH adjustment. High purity rhodamine B, fluorescein, 4-cyanobenzaldehyde, hydrazine hydrate and $PdCl_2$ are purchased from Sigma-Aldrich (India). Solvents used are of spectroscopic grade. All the metal salts used are either nitrate or chloride forms. Other analytical reagent grade chemicals are used without further purification. Milli-Q Millipore® 18.2 MΩ cm^{-1} water is used whenever required.

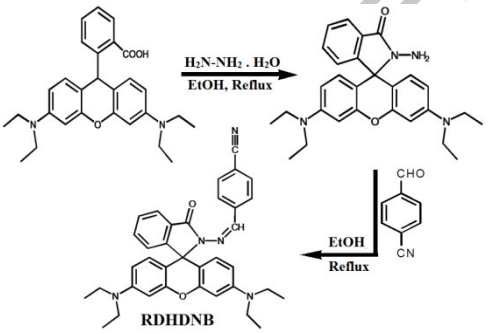
2.2. Synthesis of rhodaminehydrazone

N-(rhodamine-B) lactam-hydrazine is prepared by refluxing rhodamine B and hydrazine hydrate in ethanol.[19]

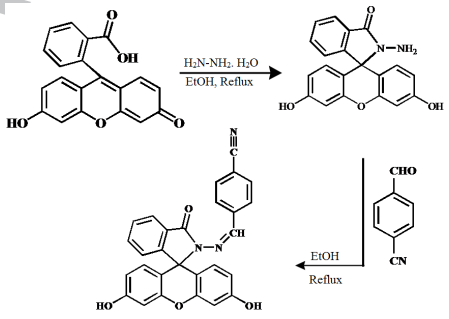
2.3. Synthesis of RDHDNB

N-(rhodamine-B) lactam-hydrazine (0.1 g, 0.2193 mmol), dissolved in 5 mL ethanol, added drop-wise to ethanol solution of 4-formylbenzonitrile (0.0287 g, 0.2193 mmol, 5 mL) under stirring condition. The reaction mixture is refluxed for 5h (Scheme 1), filtered and kept at room temperature. After 4 days, pink color rectangular crystals suitable for single crystal X-ray diffraction are collected. ^{1}H NMR (600 MHz, CDCl3) (**Fig.S1**, ESI); QTOF-MS ES+ (**Fig. S2**, ESI): $[M + H]^+$ = 570.18; FTIR/ cm−1 (**Fig. S3**, ESI): υ(C=O) 1697, υ(CH=N) 1610, υ(C≡N) 2227.

Single crystals of RDHDNB are grown from ethanol solution and characterized by X-ray crystallography (Fig.1). The selected structural parameters[20] are presented in Table S1 (ESI). The crystal structure clearly shows that two planes of the spiro compound are almost mutually vertical position.



Scheme1 Synthesis of RDHDNB



Scheme 2 Synthesis of FLHDNB

2.4. Synthesis of fluorescein hydrazide

Fluorescein hydrazide is prepared in high yield by reacting fluorescein with hydrazine hydrate in ethanol according to the literature.[21]

2.5. Synthesis of FLHDNB

FLHDNB is synthesized in a similar way to that of RDHDNB where an ethanol solution of fluorescein hydrazide (0.1 g, 0.2890 mmol) is used instead of rhodaminehydrazone (Scheme 2). ^{1}H NMR (300 MHz, DMSO-d_6) (**Fig.S4**, ESI); QTOF-MS ES+ (**Fig.S5**, ESI): $[M + Na]^+$ = 482.31; FTIR/ cm^{-1} (**Fig.S6**, ESI): υ(O-H) 3278, υ(C=O) 1680, υ(CH=N) 1610, υ(C≡N) 2225.

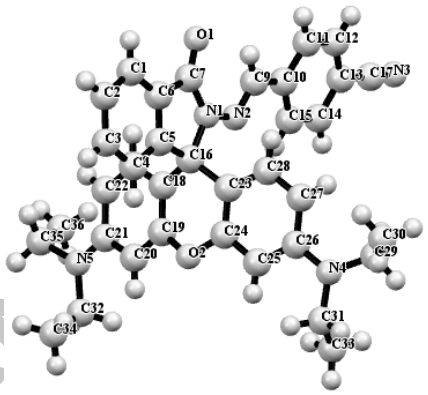


Fig.1 ORTEP view of RDHDNB (50% thermal ellipsoid probability)

2.7. *In vitro* cell imaging

Human breast cancer cell line MCF7 is grown in DMEM (Sigma, St. Louis, USA) supplemented with 10% fetal bovine serum (Sigma, St. Louis, USA), 2 mM glutamine, 100 U m/L penicillin-streptomycin solution (Gibco, Invitrogen, USA) in the presence of 5% CO_2 at 37°C. For *in vitro* imaging studies, cells are seeded in 6 well culture plates with a seeding density of 10^5 cells per well. After reaching 60–70% confluence, the previous media is replaced with serum free media, supplemented by Pd^{2+} and RDHDNB at 50 μM and 20 μM, and incubated for 2 h to facilitate the Pd^{2+} or RDHDNB uptake by the cells. Then the cells were placed under an inverted microscope (Dewinter, Italy) at different magnifications to examine any adverse effect on cellular morphology. RDHDNB treated cells are then incubated with Pd^{2+} for 15–30 min and observed under an inverted fluorescence microscope at different magnifications with a blue filter.

3. Result and discussion

3.1. Emission and absorption studies

In aqueous-acetonitrile (1: 4, v/v) medium, very weak emission of RDHDNB (λ_{em}, 586 nm; λ_{ex}, 525 nm, Φ = 0.08) increases 212 fold in presence of Pd^{2+} (Φ = 0.41) (Fig.2, (**Fig.S7**, ESI),). Very weak absorbance of RDHDNB (at 560 nm) increases with gradual addition of Pd^{2+} and the colorless solution of free RDHDNB turns pink (Fig.3, **Fig.S8**). The linear region of both the emission and absorption spectra (**Fig.S7a** and **S8a,** ESI) are useful for determination of unknown concentration of Pd^{2+}.

Plausibly, RDHDNB initially coordinates Pd^{2+} through imine nitrogen and carboxamide oxygen followed by

hydrolysis of the amide bond, releasing rhodamine B. Interestingly, these changes of optical properties are time dependent, ~40 min are required to reach a stable signal. Hence, all spectra are recorded after 40 min of mixing the ingredients. Optimization of pH is carried out to find the optimum pH for sensing Pd^{2+}. Fig.4 indicates that the entire studies can be performed at physiological pH, 7.4 (using 0.1 M HEPES buffer solution). Job's plot (**Fig.S9**, ESI) indicates 1: 1 (mole ratio) binding mode of RDHDNB with Pd^{2+}. The lowest detection limit of RDHDNB for Pd^{2+} is 5.7 × 10^{-8} M (**Fig.S10**, ESI).

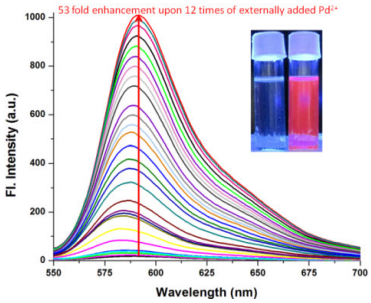


Fig.2 Changes in the emission spectra of RDHDNB (25 μM, aqueous-acetonitrile (1: 4, v/v) upon gradual addition of Pd^{2+} (0-300 μM, after 40 min of addition of Pd^{2+}). Inset pictures show the naked colors under UV light of pure RDHDNB solution and upon addition of Pd^{2+} to RDHDNB solution. λ_{ex}, 525 nm

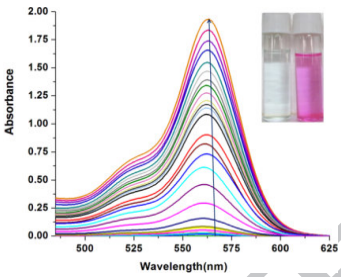


Fig.3 Changes in the absorbance of RDHDNB (25 μM, aqueous-acetonitrile (1: 4, v/v) upon gradual addition of Pd^{2+} (0- 300 μM, after 40 mins of addition of Pd^{2+}). Inset pictures show the naked colors of pure RDHDNB solution and upon addition of Pd^{2+} to RDHDNB solution.

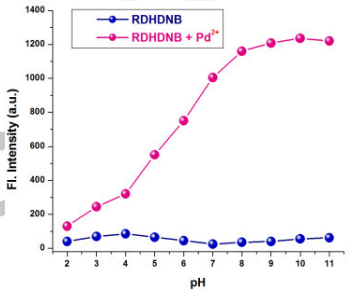


Fig.4 Effect of pH on the emission intensity of free **RDHDNB** (25 μM) and [**RDHDNB +** Pd^{2+}] system (after 40 min of addition of Pd^{2+}) (1 : 12, mole ratio, λ_{ex}, 525 nm; λ_{em}, 586 nm)

3.2. Interference and selectivity studies

No significant interference of common cations on the emission intensity of the [RDHDNB - Pd^{2+}] system has been observed (Fig.5). It is found that RDHDNB can work for visualizing Pd^{2+} well also when we use tap water as a co-solvent with acetonitrile (**Fig.S11,** ESI). Further, selectivity of RDHDNB towards Pd^{2+} is established from the emission spectra of RDHDNB in presence of equimolar amount of common cations and anions separately (Fig.6). Corresponding color changes observed under UV light and naked eye are shown in **Fig.S12** (ESI). To demonstrate the practical application of RDHDNB, paper strips coated with sensor is prepared by soaking the filter paper into the solution of RDHDNB (25 μM) followed by drying in air. Next, the so prepared paper strips are soaked in solutions having different Pd^{2+} concentration, *viz.* 0 M, 1.0 ×10^{-5} M, 5.0 ×10^{-5}, 1.0 ×10^{-4} M and 3.0 ×10^{-4} M. **Fig.S13** shows the colors of the test strips that changes from colorless to pink and deepens gradually with the increasing Pd^{2+} concentration. This technique may be useful as a simple tool for detecting Pd^{2+} in environmental samples.

Although the reference compound, FLHDNB contains same chelating sites remain silent towards Pd^{2+}, monitored using both absorption and emission spectroscopy (**Fig.S14**, ESI). Probably, weak electron donation ability of –OH group of fluorescein than the $-N(Et)_2$ of rhodamine B is responsible for the difference.

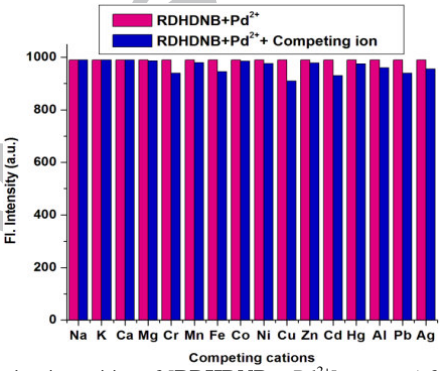


Fig.5 Emission intensities of [**RDHDNB +** Pd^{2+}] system (after 40 mins of addition of Pd^{2+}) in presence of competing cations

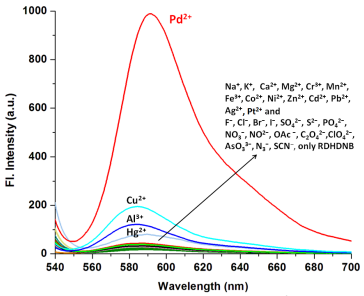


Fig.6 Emission spectra of **RDHDNB** (25 μM, λ_{ex},525 nm) in aqueous-acetonitrile (1: 4, v/v) solution in presence of different ions (12 equiv., after 40 mins of addition of different ions)

4. Plausible sensing mechanism

Mass spectrum of the [RDHDNB - Pd^{2+}] system (**Fig.S15**, ESI) provides a reasonable evidence for plausible Pd^{2+} sensing mechanism of RDHDNB. The molecular ion peak at m/z = 443.40 corresponds to rhodamine-B, also corroborated from its FTIR spectrum (**Fig.S16**, ESI) having υ(C═O), 1336 cm^{-1}. These clearly indicate Pd^{2+} assisted hydrolysis of RDHDNB (Fig.7). The breaking of amide bond releases the corresponding hydrazone of 4-formylbenzonitrile, $C_8H_7N_3$, which remains complexed with Pd^{2+} along with other coligands like hydrazine (N_2H_4) and chloride ions (m/z, 361.52 ([$C_8H_7N_3$+N_2H_4+2Cl^-+Li^+]$^+$). The hydrazone unit further raptures at imine functionality to release 4-formylbenzonitrile (C_8H_5NO, m/z, 132.16

($[C_8H_5NO+H]^+$). Thus, Pd^{2+} is not released in the solution, thus cannot be termed as catalyst. .

Time dependent NMR spectra before and after 40 min of addition of Pd^{2+} to RDHDNB show a new peak at 9.9 ppm corresponding to the aldehyde proton of 4-formylbenzonitrile appears (Fig. 8). All the other protons of RDHDNB downfield shifted after interaction with Pd^{2+}.

Additionally, to examine any reversibility of the emission spectral signals, excess disodium salt of ethylenediamine tetraacetic acid ($EDTANa_2$) is added as Pd^{2+} chelating agent to the color solution of [RDHDNB - Pd^{2+}] system. However, no change of color and emission intensity of the solution is observed that indicates the irreversible feature of the recognition process.

A control experiment for sensing of Pd^{2+} in anhydrous acetonitrile medium instead of aqueous-acetonitrile (1: 4, v/v, 0.1 M HEPES buffered, pH, 7.4) solution is carried out without success. This indicates that presence of water is essential for Pd^{2+} recognition event which further hints hydrolysis event. All these results affirm the proposed Pd^{2+} sensing mechanism as portrayed in Fig. 7.

The kinetics of the Pd^{2+} recognition event suggests a two-step reaction mechanism. The first step i.e., the chelation of Pd^{2+} is fast ($k_1 = 3.65 \times 10^{-3}$) and the second step i.e., the hydrolysis step is slow ($k_2 = 3.10 \times 10^{-4}$) (**Fig.S17**, ESI). These results are also in line to our proposed Pd^{2+} sensing mechanism.

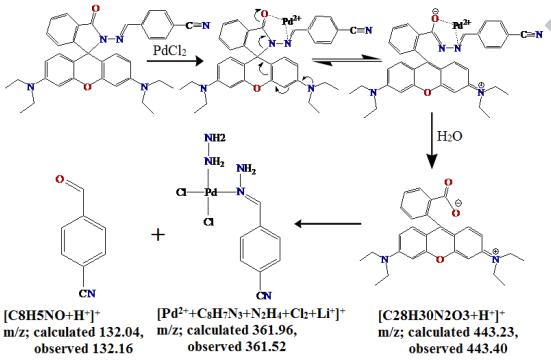


Fig.7 Proposed Pd^{2+} sensing mechanism

5. Cell imaging

RDHDNB is capable to detect intracellular Pd^{2+} in human breast cancer cells, MCF7 under fluorescence microscope. Cells treated with RDHDNB (without Pd^{2+}) are used as control. Fig.9 indicates that RDHDNB is able to permeate cell membrane to stain Pd^{2+} without any harm (cells remain alive even after several hours of exposure to 20 μM RDHDNB).

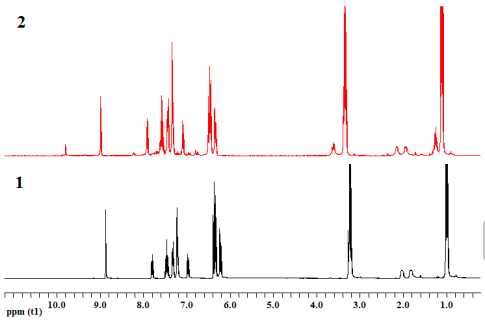


Fig.8 ^1H NMR spectra of RDHDNB (1) and [RDHDNB - Pd^{2+}] system recorded after 40 min of addition of Pd^{2+} (2) in $CDCl_3$.

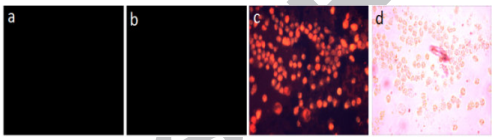


Fig.9 Fluorescence microscope images of MCF7 cells: (a) and (b) are images of cells after 2 h incubation with Pd^{2+} (50 μM) and RDHDTNB (25 μM) respectively, while (c) shows cells after 2 h incubation with 50 μM Pd^{2+} followed by addition of 20 μM RDHDNB solution and (d) is the bright field image of the cells after incubation with 20 μM RDHDNB for 2h followed by the addition of 50 μM Pd^{2+}

6. Conclusion

Rhodamine B hydrazide derived probe, RDHDNB functions as an excellent colorimetric and fluorescence probe for trace level determination of Pd^{2+} up to 5.7×10^{-8} M. Chelation followed by hydrolysis of the amide bond releases the rhodamine B unit, responsible for Pd^{2+} sensing at physiological pH. The probe can detect intracellular Pd^{2+} in human breast cancer cells, MCF7 under fluorescence microscope.

Acknowledgement

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CCDC number of **RDHDNB** is 948697. ESI is available.

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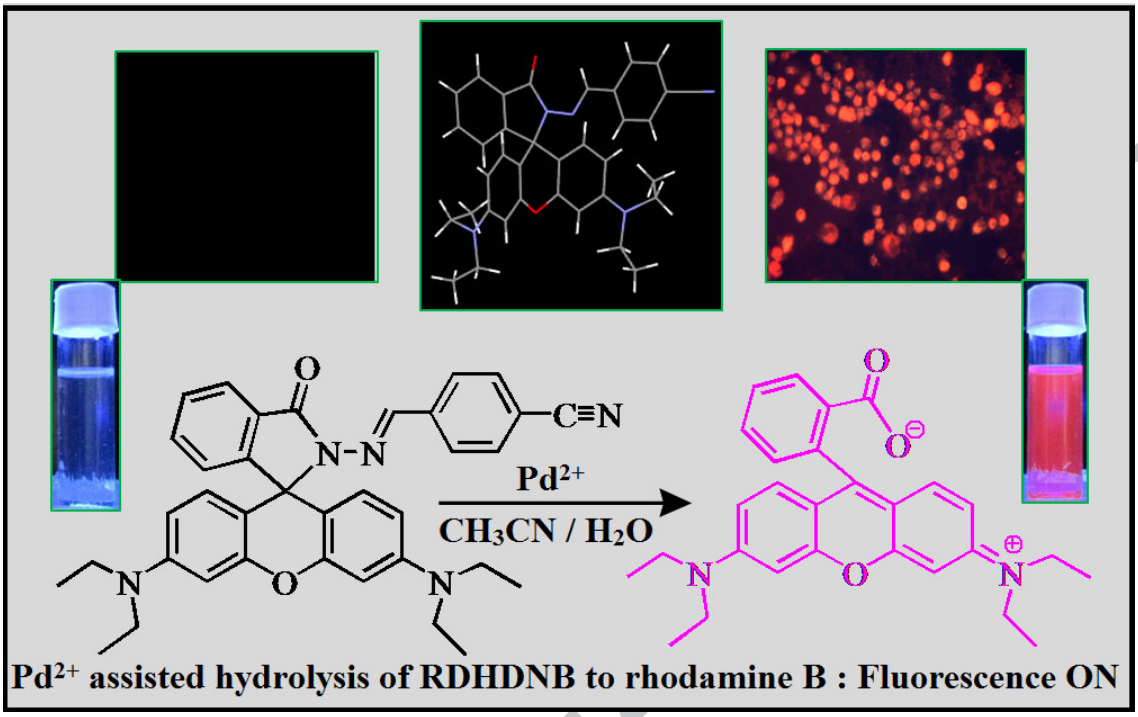

4-Benzonitrile appended rhodamine B hydrazide derivative (RDHDNB) selectively detects Pd^{2+} both naked eye and under UV light. Moreover, RDHDNB easily detects Pd^{2+} in human breast cancer cells MCF7, under normal and fluorescence microscope.

Highlights

1. Visible light excitable, single crystal X-ray structurally characterized Pd^{2+} selective fluorescence probe.

2. Lowest detection limit for 5.7×10^{-8}M

3. RDHDNB is useful to detect Pd^{2+} in human breast cancer cells MCF7, under normal and fluorescence microscope.

4. To the best of our knowledge, this is the first report on Pd^{2+} assisted hydrolysis of a rhodamine based fluorescence probe, RDHDNB for its colorimetric and fluorescence detection.

5. Common ions do not interfere.