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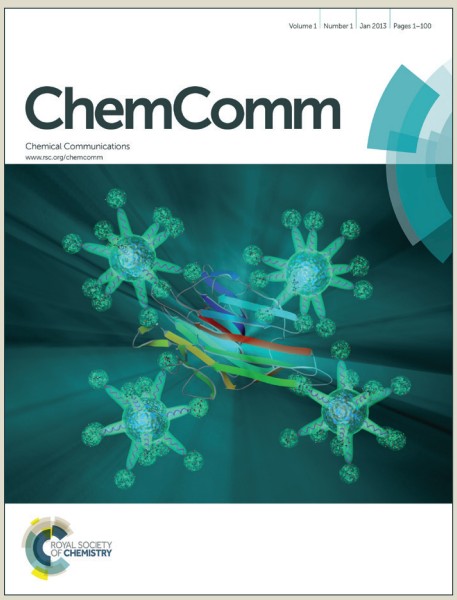

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A new turn on Pd^{2+}-specific fluorescence probe and its use as an Imaging reagent for cellular uptake in Hct116 cells

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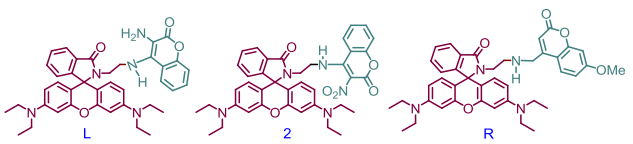
A new coumarin-rhodamine conjugate is used as a specific probe for Pd^{2+} ion and this could even delineate Pd(II) from Pd(0) or Pd(IV) in aqueous buffer medium (pH ~7). Laser confocal microscopic studies reveal that efficient cellular internalization of this reagent helps in imaging the cellular uptake of Pd^{2+} as low as 0.1 ppm in Hct 116 cells. This reagent could even be used for estimation of Pd^{2+} in human urine sample.

Palladium has found wide applications in organic synthesis, drug design, pharmaceuticals and commercial materials such as fuel cells, dental crowns, medical instruments, jewellery etc., owing to its inertness, biocompatibility and versatility as a catalyst.[1] Role of diverse Pd-complexes in different coupling reactions is explored extensively by synthetic chemists as well as for production of a range of crucial fine chemicals and drug molecules.[2] Despite exhaustive purification processes, certain amount of palladium could remain as an impurity in the final product,[2b,3] which is consumed along with the drug. Previous reports reveal that palladium has the propensity to coordinate to DNA, certain proteins/ thiol-containing amino acids, and vitamin B6, and disrupt some cellular processes, thereby leading to health problems.[4] Apart from consumption of certain drugs/pharma products that are contaminated with trace amount of palladium ion, human population in general gets exposed to palladium through dental alloys, jewellery, food and emissions from automobile catalytic converters. Average dietary intake of palladium for an adult is estimated as ~ 1.5–15 μg/day as per WHO report and its permitted threshold level in drugs is 5-10 ppm.[3] It has been argued that excretion of palladium happens mostly through urine and its concentration in urine typically lies in the range of 0.006 - <0.3 μg/l in adults.[5] All these have necessitated the development of an efficient fluorescent probe that could bind specifically and reversibly to Pd(II) ion, the most abundant oxidation state for palladium metal that could exist in physiological conditions or in live cells. Such a molecular probe could be ideal for detection of cellular uptake of Pd(II) or for diagnostic application. Apart from these, reagent that also allows instantaneous infield analysis of environmental sample has a distinct edge over other analytical methods and procedures. Conventional methods for palladium detection rely mostly on analytical methodologies like atomic absorption spectrophotometry, solid-phase micro extraction-high-performance liquid chromatography and ion-coupled plasma emission-mass spectrometry. Such procedures are generally expensive, time-consuming and need highly skilled individuals.[6] Such methodologies also involve complicated sample preparation procedures. These limitations have further exemplified the scope of an efficient and sensitive molecular probe that allows *turn-on* fluorescence response on specific binding to Pd(II) ion in aqueous buffer medium having physiological pH as well as an imaging reagent.[7] Pd(II) is generally known to be an efficient quencher for luminescence, as this being a heavy-atom favours an effective spin-orbit coupling and a non-radiative deactivation of the excited states.[8] Thus, example of the *fluorescence off*-based receptors for palladium is abundant in contemporary literature.[8] Among limited *fluorescence on*-based receptors reported in the literature,[9] very few have been utilized either as an imaging reagent or for detection of palladium in biological fluids.[10] More importantly, none of such receptors are found to be specific towards Pd(II).[8f,9i,j,10h-j] This motivated us to develop a rhodamine based molecular probe (**L**) that was specific towards Pd(II) among all other metal ions in physiological condition and could be utilized also as an imaging reagent or for detection of Pd(II) present in biological fluids like human urine sample. Conversion of the cyclic lactam form (*Fluorescence-Off* mode) to the acyclic xanthenes form (*Fluorescence-On* mode) of a suitably substituted rhodamine derivative (**L**) on specific binding to Pd(II) has been described in the present article. We could demonstrate that this new molecular probe could bind to Pd(II) in presence of all other common metal ions, including Pd(0) or Pd(IV), under physiological condition with an associated *switch on* colorimetric as well as luminescence response. Further, this non-toxic reagent could permeate the cell membrane of Hct116 cells and be utilized for detection of Pd(II) in human urine sample. Such a reagent that allows instant and reversible responses is generally preferred over chemodosimetric reagents, which usually need certain incubation time for completion of the reaction and thus, the recognition process.[11]



Scheme 1 Molecular structures for **L** as well as for two model reagents **2** and **R**.

Detailed synthetic procedures for the probe molecule **L**, its various analytical and spectroscopic characterization data are provided in the supporting information.† All such data ensured the desired purity for **L**. Uv-vis and luminescence spectra for **L** (10 μM) were recorded in aq. HEPES buffer (10 mM)-acetonitrile (1:1, v/v; pH 7.2) medium (Fig. 1).

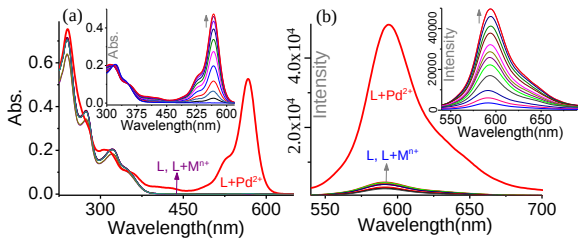
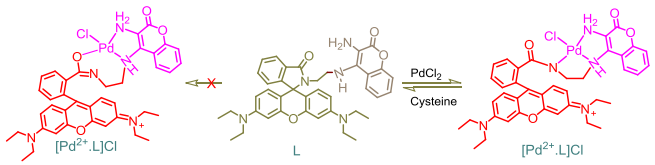


Fig 1. Changes in (a) Absorption and (b) Emission spectra (λ_{Ext} of 530 nm; slit = 2/2 nm) of the receptor **L** (10 μM) in absence and presence of 100 μM different metal ions (M^{n+} = Li^+, Na^+, K^+, Mg^{2+}, Al^{3+}, Ca^{2+}, Ba^{2+}, Sr^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+}, Cd^{2+}, Co^{2+}, Fe^{2+}, Fe^{3+}, Cr^{3+}, Pb^{2+}, Pd^{2+}, Pt^{2+}, Pd^0, Pd^{4+}); Insets (a): Systematic changes in (a) absorption and (b) emission (λ_{Ext} = 530 nm; slit width 2/2 nm) spectral pattern for **L** (10 μM) in presence of varying [Pd^{2+}] (0.0 – 20 μM); All studies were performed in aq. HEPES buffer-acetonitrile (1: 1, v/v; pH 7.2) medium.

As anticipated for spirolactam derivative, reagent **L** did not show any absorption or emission spectral band beyond 400 nm and this accounted for it colorless solution at pH = 7.2. A characteristic signal at ~ 65.43 ppm for the tertiary C-atom in the ^{13}C NMR spectrum for **L** also confirmed its spirolactam structure.[12] Absorbance spectra of **L** (10 μM) were recorded in absence and presence of 100 μM of various metal ions (Na^+, K^+, Ag^+, Hg^{2+}, Pb^{2+}, Cd^{2+}, Cu^{2+}, Cr^{3+}, Ni^{2+}, Fe^{3+}, Co^{3+}, Zn^{2+}, Ca^{2+}, Al^{3+}, Pt^{2+} and Mg^{2+} including Pd^0 and Pd^{4+} in aq.buffer-acetonitrile media (pH 7.2) (Fig 1a). Among all these cationic analytes, a distinct new spectrum having a band maximum at 567 nm appeared only in presence of Pd^{2+} and this accounted for the visually detectable change in solution colour from colourless to purple. Further, interference studies in presence of 10 mole excess of all other metal ions, including Pd^0 and Pd^{4+}, ensured that the reagent, **L** was specific towards Pd^{2+} even in presence of competing cationic analytes.(SI Fig.16) For similar experiments with fluorescence measurement, revealed appearance of an emission band with maxima at 594 nm (Fig. 1b) using λ_{Ext} of 530 nm. No such change or insignificant changes was/were observed for all other cations that we studied, including Pd^{4+} or Pd^0. These results revealed that apart from Pd^{2+}, all other metal ion (including Pd(0)) either failed to bind to **L** or a binding of **L** to any of these metal ions were too weak to convert the non-luminescent spirolactam derivative to the acyclic and luminescent xanthenes form.[12] Results of the ^1H & ^{13}C NMR studies further confirmed this. The emission quantum yield for **L** was evaluated as ϕ_L = 0.011 for λ_{Ems} of 594 nm (λ_{Ext} = 530 nm), while this value was found to be 0.245 in presence of Pd^{2+}. Peng and his co-workers have contributed significantly in developing efficient and specific receptor for Pd(II) species. However, most of his receptors for Pd(II) also showed sluggish but similar responses towards Pd(0).[9b] In few instances, receptors for Pd(II) showed distinct interference of Pd(IV).[8f,9i,j,10h-j] To the best of our knowledge, this present molecular probe is the first example of a receptor that showed specificity towards Pd(II) (Fig. 1) in physiological condition.

It may be noted that the electronic/luminescence spectral changes as well as the visually detectable changes in solution colour/luminescence for **L** were instantaneous on binding to Pd(II). Importantly, changes in the intensity of the absorption band at 567 nm and emission band at 594 nm showed a linear dependency on [Pd^{2+}] over a concentration range of 1.0 to 20 μM. B-H plots, obtained by using data available from systematic absorption and emission spectral titrations (at 25°C) (Fig 1a,b), revealed association constant of (4.8 ± 0.5) x 10^3 M^{-1} and (5.8 ± 0.5) x 10^3 M^{-1}, respectively in aq. buffer-CH_3CN (1:1, v/v; pH = 7.2) medium. Binding stoichiometry of 1:1 was ascertained from the good linear fit of the B-H plot.(SI Fig.11) Further, this binding ratio was confirmed from the results of the ESI-Ms data; a signal at m/z 784.63 was attributed to [{Pd^{2+}.L}Cl^-].(SI Fig.9) Lowest detection limit (3σ/slope) for Pd^{2+} detection using data available from fluorescence titration and was found to be 0.106 ppm.[13] Reversibility of the binding process between **L** and Pd^{2+} was established by allowing Pd^{2+} to form a even more stable complex Pd(Cys)$_2$ (Cys is Cysteine and $K_{Association}$ for Pd(N-acetyl cys)$_2$ is reported to be ~ 10^8).[14] Purple solution of Pd^{2+}.**L** turned colourless on addition of 2 mole equiv. of Cys; both electronic as well as the luminescence spectra for **L** was restored in the visible region.(SI Fig.17)



Scheme 2 Proposed mechanism for Pd^{2+} complexation inducing the opening of the spirolactam ring in **L**.

The luminescence *switch ON* response of the reagent **L** happened at pH 7.2 only on specific binding to Pd(II), which led to the transformation from a non-luminescent cyclic lactam form to an acyclic luminescent xanthenes form. Under the similar media pH (7.2), reagent **L** alone remained in the non-luminescent lactam form. UV-vis and luminescence spectra recorded for **L** (10 μM) at varying solution pH and results of such studies revealed that the conversion from spirocyclic to acyclic xanthenes form of the reagent **L** alone could happen only for pH ≤ 4.5.(SI Fig.13) This confirmed that luminescence on response at ~590 nm was only due to the specific binding of the reagent **L** to Pd(II). Most importantly, none of the metals including Pd^0 and Pd^{4+} could influence the optical response of **L** on binding to Pd^{2+}.

Emission spectra recorded for **L** (with λ_{Ext} = 360 nm) showed significant luminescence quenching for the coumarin moiety (λ_{Ems}^{Max} at 450 nm) in presence of Pd(II) and an insignificant spectral overlap with the absorption spectral band with maxima at ~567 nm for Pd^{2+}.**L** nullified the possibility for any free resonance energy transfer process (FRET).(SI Fig.15) In two of our previous reports we have shown that Hg(II)–η^2-arene π-interaction in a rhodamine-coumarin hybrid could induce a through bond energy transfer (TBET) process.[15] Pd(II) is also known to form stable M^{n+}–η^2-arene π bond. Presumably, coordination

of the 3-amino functionality of the coumarin moiety did not favour such an interaction. In absence of such an interaction the possibility of any TBET process was abolished. Model reagent **R** was synthesized following literature report to unveil the crucial role that this 3-amino functionality played in binding to Pd^{2+} ion. All relevant characterization data for **R** are provided in the supporting information section. UV-vis and fluorescence spectra recorded for **R** did not show any detectable change on addition of Pd^{2+} under the identical experimental condition. (SI Fig.14) This corroborates that amine group of **L** played a pivotal role in the fast and specific binding to Pd^{2+} ion.

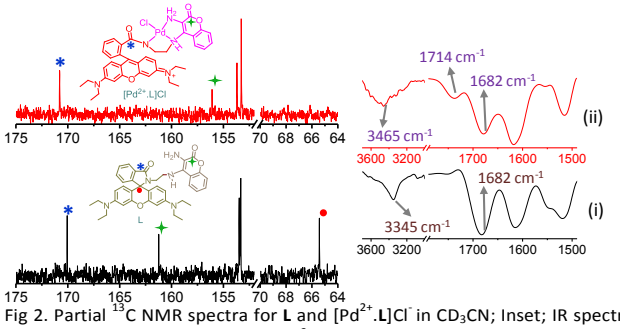


Fig 2. Partial ^{13}C NMR spectra for **L** and $[Pd^{2+}.L]Cl^-$ in CD_3CN; Inset; IR spectra of **L** in (i) absence and (ii) presence of Pd^{2+}.

Stretching frequencies for C=O group in FTIR spectra of the coumarin moiety in **R** and **2** (ESI for detailed characterization data for **2**) appeared at ~1714 and ~1719 cm^{-1}, respectively.(SI Fig.4&10) The absence of such a band for **L** could be ascribed to a strong hydrogen bonding interaction between $O_{C=O}$ of the coumarin moiety and $H_{3\text{-amino}}$ functionality, which induced a shift to higher energy and eventually got masked within the strong band at ~1685 cm^{-1}, the stretching band for the $C=O_{Amide}$ of the rhodamine moiety. This band appeared almost in the similar frequency region for all three rhodamine derivatives (e.g. **R**, **2** and **L**) and it remained almost invariant on binding of **L** to Pd^{2+}. This tend to leave us with an impression that $O_{C=O[Amide]}$ of the rhodamine moiety was not involved in coordination to Pd^{2+}-centre in Pd^{2+}.**L**. ^{13}C NMR spectra recorded for **L** confirmed that that band for $C_{C=O}$ of coumarin moiety was up field shifted by 5 ppm ($-\Delta\delta \sim 5$ ppm; 161 ppm to 156 ppm) on binding of **L** to Pd^{2+}, while insignificant shift ($\Delta\delta \sim 1$ ppm; 161 ppm to 162 ppm) for $C_{C=O[Amide]}$ of rhodamine moiety was observed (Fig 2). This further corroborated our presumption that $O_{C=O[Amide]}$ of the rhodamine moiety was not involved in coordination to Pd^{2+} either in keto form or in enolate form.[10i] FTIR spectra also revealed that the N-H stretching frequency for the $-NH_2$ functionality became broad and shifted to lower energy (by ~120 cm^{-1}) on binding of **L** to Pd(II). Based on these and the results of the mass spectral data coordination mode for Pd^{2+}.**L** was proposed (Scheme 2).

As stated earlier, major route for the excretion of Pd^{2+} is through urine. Thus, design of an appropriate reagent capable of detecting Pd^{2+} in human urine sample, is crucial. The Pd^{2+} levels in adult human urine sample typically lies in the range of 0.006 - < 0.3 μg/litre.[5] To check the possibility of using new reagent **L** for such an application and evaluating unknown $[Pd^{2+}]$ in urine, a calibration curve was generated. A methodology for generation of the calibration curve (ΔI; I is change in emission intensities) vs. known $[Pd^{2+}]$ (0 to 7 μM) with the linear fluorescence response range for estimation of unknown Pd^{2+} in mixed aq. buffer (pH 7.2) solution (Fig. 3) are discussed in detail in ESI. The final [**L**] of 10 μM was used for estimation of Pd^{2+} in urine sample. Urine sample was appropriately diluted (100 times with aq. HEPES buffer: acetonitrile (1:1, v/v) mixture) and used for estimation of Pd^{2+} without further treatment before use.

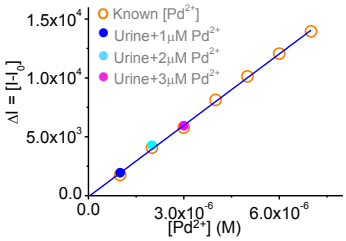


Fig 3. Plot of ΔI (I_0 - I) vs. $[Pd^{2+}]$, where I_0 and I are emission intensities of receptor **L** at 594 nm (λ_{Ext} = 530 nm) in the absence and presence of known $[Pd^{2+}]$ and urine sample spiked with a known $[Pd^{2+}]$.

Some of those solutions were spiked with known $[Pd^{2+}]$ (1 μM, 2 μM and 3 μM) as an internal standard. The Pd^{2+} concentration in urine was determined to be 0.2 μg/litre, which is within the allowed limit for Pd^{2+} content in adults urine sample.[5]

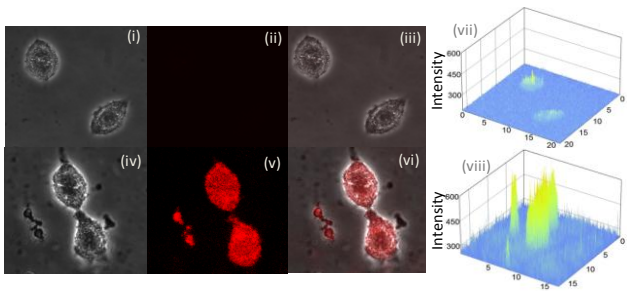


Fig 4. (i)-(iii) Confocal laser scanning microscopic (CLSM) images of Hct 116 colon cancer cells incubated with **L** (10 μM) as control: (i) bright field image, (ii) images observed in red channel and (iii) overlay image of (i) and (ii); (iv-vi) CLSM images of Hct116 cells incubated with **L** (10 μM) for 30 min and then further exposed to Pd^{2+} (0.1 ppm) for 30 min at 37 °C: (iv) bright field images, (v) images observed in red channel and (vi) overlay images of (iv) and (v); Graphical 3D CLSM images of Hct116 cells (vii) pre-exposed to **L** (10 μM) only and (viii) pre-treated with **L** (10 μM)) for 30 min and then further exposed to a Pd^{2+} (0.1 ppm) (λ_{Ext} = 530 nm).

Further, the possibility of using this molecular probe as an imaging reagent for detection of cellular uptake of Pd^{2+} was explored. MTT assay studies revealed that this reagent showed insignificant toxicity towards Hct116 cells.(SI Fig.21) Then, this reagent was used for the detection of Pd^{2+} uptake in live cancer Hct116 cells using a laser CLSM studies following excitation at 530 nm. Initially, live Hct116 cells were incubated with only **L** (10 μM) for 30 min at 37 °C. After necessary and thorough washing, these cells showed no fluorescence and these images were used as control. Further, CLSM images showed that the Hct116 cells that were pre-treated with 10 μM reagent **L** (and thoroughly washed for removal of the surface adhered reagent) and subjected to a follow-up treatment with Pd^{2+} (0.1 ppm) led to a strong fluorescence in the red channel (Fig. 4). interestingly, figure 4 also reveals that reagent **L** is completely localised within the cell. These results demonstrated two important aspects: reagent **L** was cell membrane permeable and could be used as an imaging reagent for the detection of Pd^{2+} uptake in living cells. These results also confirmed that the uptake of Pd^{2+} as low

as 0.1 ppm, which is drastically lower than the specified threshold in drugs (5-10 ppm), could be detected in live Hct116 cells from confocal images.

Conclusions

In conclusion, we have synthesized a new coumarin appended rhodamine based fluorescent probe **L**, which showed a *Turn ON* fluorescence response on specific binding to Pd^{2+} in an ensemble of several other metal ions. This reagent was found to be non-toxic to live Hct116 cells and was cell membrane permeable. This allowed this reagent to be used as an imaging reagent for detection of the cellular uptake of Pd^{2+} ion in live Hct116 colon cancer cell lines as well as to be used for estimating Pd^{2+} in human urine sample. Such an example is scarce in the contemporary literature.

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Notes and References

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