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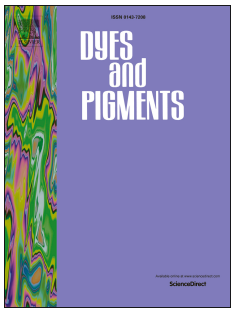

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Sugandha Dhoun, Ishpreet Kaur, Paramjit Kaur, Kamaljit Singh

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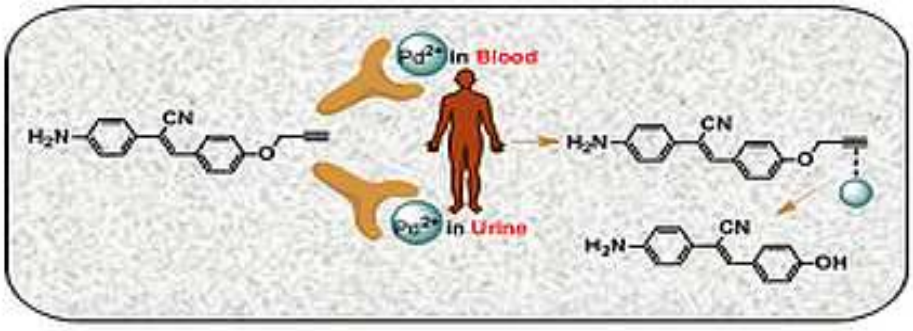
Sugandha Dhoun, Ishpreet Kaur, Paramjit Kaur* and Kamaljit Singh*

Department of Chemistry, UGC Centre of Advanced Studies-II, Guru Nanak Dev University,

Amritsar- 143 005, India.

E-mail: paramjit19in@yahoo.co.in; kamaljit.chem@gndu.ac.in



An α-cyanostilbene based chemodosimeter for the detection of Pd^{2+} with application in urine sample and human blood serum.

Propargylated cyanostilbene based chemodosimeter for Pd^{2+} with application in biological fluids

Sugandha Dhoun, Ishpreet Kaur, Paramjit Kaur* and Kamaljit Singh*

Department of Chemistry, UGC-Centre for Advance Studies-II, Guru Nanak Dev University, Amritsar 143005, India

Corresponding author: Tel. +91 183 2258853, Fax. +91 183 2258819-20

E-mail: *paramjit19in@yahoo.co.in* (P. Kaur); *kamaljit.chem@gndu.ac.in* (K. Singh)

Abstract: A new propargylated cyanostilbene has been synthesized and was found to be sensitive to Pd^{2+} ions over the other metal ions tested, with a significantly lower detection limit of 9.3 ppb under mild conditions. The sensing protocol proceeds via palladium catalyzed depropargylation reaction. The probe was also explored for its potential to detect Pd^{2+} in human urine and blood samples. The noted significant % age of spiked recoveries in both cases depict promising potential of **3** for practical application. Importantly, the reported sensing protocol is first time employed for the detection of Pd^{2+} ions from biological fluids.

Keywords: chemodosimeter, palladium ion, depropargylation, application in urine, blood serum samples.

1. Introduction

Palladium (Pd^0 or Pd^{2+}) is one of the most widely used metal ions in metal supported catalysts in current times and is employed in many coupling and/or cross-coupling reactions [1-5] such as Heck reaction between alkenes and aryl halides, Suzuki reaction between aryl halides and

boronic acids, Stille reaction of organohalides and organotin compounds, Negishi coupling of organohalide and organozinc compounds, Sonogashira coupling with copper(I) iodide as additional catalyst, Kumada coupling of Grignard reagents with aryl or vinyl halides, Nazarov-type cyclization reactions, Suzuki-Miuaura, Buchwald-Hartwig reactions, palladium catalyzed, cyanation, monocarbonylation, oxidative conversion of alcohols to aldehydes, C-H functionalizations, including synthesis of many drugs e.g. roficoxib and cofprozil etc. However, its wide application range is eclipsed by the adverse influence on human health and environment, especially when the end products of such chemical processes are contaminated with palladium [6]. In such cases the much necessitated extensive purification of the contaminated products leads to the release of palladium into the environmental resources and biological systems, thus posing serious threat to the human health [7]. The palladium ions can bind to thiol-containing amino acids, proteins, DNA and RNA and may disturb various cellular processes, even when present in extremely low doses [8-10]. In order to check such hazards, the environment protection agencies have strictly limited its concentration to 5-10 ppm in the end products of the reactions [11].

Among the various oxidation states of palladium (0, +2, +4), the species with +2 oxidation (the most abundant) state are the most toxic. Consequently, chemists, biologists, clinical biochemists and environmentalists in recent years have shown great interest in the development of receptors for palladium [12-16]. These receptors are more often coordinating ligands with heteroatoms such as sulfur and nitrogen atoms as binding sites. The recognition of Pd^{2+} is manifested in the form of perturbations in their absorption/emission behaviours [17-19]. However, many of them suffer limitations of selectivity because hetero atoms may exhibit affinity for other metal ions in addition to palladium ions. Consequently, literature has witnessed a number of reports, which

rely upon the metal ion-specific chemical reactions such as Pd^{2+} catalyzed oxidative cyclization reaction, deallylation reaction, depropargylation reaction etc. [20-29], which indeed have alleviated the selectivity issues, otherwise associated with the former type of receptors. In one of our earlier investigations [30], we also encountered the selectivity issue where the BODIPY-dithia-dioxa-azacrown conjugate was found to be sensitive to both Pd^{2+} and Hg^{2+} ions. However, performing the experiments in the presence of cysteine allowed detection of Pd^{2+} in such mixtures. Thus, we sought to develop a probe, which could work as a chemodosimeter through reaction with Pd^{2+} ions. Utilizing the facility of Pd catalyzed cleavage of terminal propargyl ether the cyanostilbene probe **3** bearing a terminal propargyl ether moiety (Scheme 1) was synthesized.

Previous work [31-36] utilizing this strategy has shown great advantages in terms of sensitivity and selectivity, however, the use of demanding experimental conditions w.r.t. time, nature of solvent, heating etc. has been a matter of concern in the direction of efficient and mild sensing conditions. The present probe has shown the advantage as it works in mild conditions (aqueous medium, absence of base or any other reagent), which is an added advantage as far as its possible implementation in the physiological conditions is concerned, besides significantly low calculated detection limit.

Furthermore, without using any additional reagent, **3** was successfully implemented for the detection of palladium in the human urine, which is one of the sources of palladium contamination in the environment [37]. As per our knowledge, this is the first report on the detection of palladium in urine sample *via* depropargylation mechanism. Additionally, we also explored its utility for the detection of palladium (Pd^{2+}) in human blood serum, as it is the most toxic state in which Pd exists.

2. Experimental

2.1. Materials and reagents

All liquid reagents were dried/purified following recommended drying agents and/or distilled over 4 Å molecular sieves. Toluene was dried using sodium benzophenone ketyl. DMF and 1,4-dioxane were stored overnight over molecular sieves. 4-nitrophenylacetonitrile was bought from SIGMA ALDRICH and used as such. 4-hydroxybenzaldehyde, propargyl bromide, piperidine, sodium hydride were purchased from Spectrochem where as sodium sulfide was purchased from SD-fine and used as received. The solution of $PdCl_2$ was prepared in 3:1 brine/MeOH. The solutions of all other cations and anions were prepared in distilled water.

2.2. Instrumentation

Fluorescence studies were carried out using Perkin Elmer LS 55 Fluorescence Spectrometer. UV-visible studies were carried out using UV-1800 SHIMADZU UV-Spectrophotometer. The pH titrations were carried out using Equip-Tronics Digital pH meter model –EQ 610 and electrode was calibrated using standard buffers of pH 4.0, 7.0 and 9.2. Infrared spectra were recorded on Perkin-Elmer FTIR-C92035 Fourier-transform infrared (FT-IR) spectrophotometer in range 400–4000 cm^{-1} as KBr pellets. ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker Biospin Avance III HD at 500 MHz, with TMS as internal standard using $CDCl_3$, DMSO-d_6 and D_2O as deuterated solvent. Data are reported as follows: chemical shift in ppm (δ), integration, multiplicity (s = singlet, d = doublet, t = triplet, m = multiplet), coupling constant (*J*) in Hertz (Hz). Mass spectrum (MS) was recorded on Bruker HRMS MICROTOF II spectrometer. Electron diffraction (ED) pattern and transmission electron microscopic (TEM) images were

recorded on a JEOL JEM 2100 HRTEM instrument. Dynamic Light Scattering was carried out using MALVERN Zetasizer Nano ZS instrument.

2.3. Quantum yield calculation

The fluorescence quantum yields were measured with respect to quinine sulfate [38] as standard having quantum yield of 0.54 in 0.1 M H_2SO_4.

$$\Phi_u = \Phi_s \cdot F_u.(1\text{-}10^{-AsLs}).n_u^2/(1\text{-}10^{-AuLu}).\ F_s.A_u.n_s^2$$

Φ = quantum yield, F = Integrated fluorescence intensity, A = Absorbance, n = refractive index of solvent, L= length of cell. (1.0 cm for standard and sample), s = standard i.e. quinine sulfate u = sample

2.4. Detection limit calculations

The detection limit was calculated on the basis of the change in fluorescence intensity at 517 nm after addition of minimum amount of Pd^{2+}. The fluorescence emission spectrum of **3** was measured 6 times, and the standard deviation of blank measurement was achieved. The detection limit was calculated using the following equation.

$$\text{Detection limit} = 3 \times \sigma/K$$

σ = Standard deviation of blank measurement, K = Slope between the ratio of emission intensity versus [Pd^{2+}]. The detailed calculations are shown on S16.

2.5. Computational details

All theoretical calculations were carried out by using the Gaussian 09 suite of programs [39] The molecular geometries of the chromophores were optimized at the DFT method employing the

hybrid B3LYP functional and 6-31G basis set and LanL2DZ basis set in case of palladium metal ion. The molecular orbital contours were plotted using Gauss view 5.0.9.

*2.6. Procedure for synthesis and characterization of **3** (Scheme-1)*

*2.6.1 Synthesis of (Z)-3-(4-hydroxyphenyl)-2-(4-nitrophenyl)acrylonitrile **1***

To a mixture of the 4-hydroxybenezaldehyde (2.0 g, 16.37 mmol) and 4-nitrophenylacetonitrile (2.66 g, 16.37 mmol) in absolute EtOH (40 ml), was added piperidine (2.43 ml, 24.58 mmol) portion-wise and stirred at room temperature for 3 h. After completion (TLC), the reaction mixture was cooled to 0 °C and filtered to obtain precipitates, which were further washed with EtOH and dried under vacuum to obtain analytically pure dark red solid **1** (95%). IR (KBr) ν_{max} : 1163, 1338, 1434, 1502, 1553, 1598, 2206, 2517, 2927, 3183 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$, 25 °C) δ (ppm): 7.58 (t, *J* = 5 Hz, 3H, ArH), 7.62 (d, *J* = 5 Hz, 2H, ArH), 8.02 (d, *J* = 5 Hz, 2H, ArH), 8.33 (d, *J* = 10 Hz, 2H, ArH). ^{13}C NMR (125 MHz, $CDCl_3$, 25 °C) δ (ppm): 114.95, 116.70, 124.24, 124.72, 127.73, 129.98, 132.41, 132.54, 139.20, 139.31, 148.39. HRMS: *m/z* calculated for $C_{15}H_{10}N_2O_3$: 267.0764 and found: 267.0730 (M^++1).

*2.6.2 Synthesis of (Z)-2-(4-aminophenyl)-3-(4-hydroxyphenyl)acrylonitrile **2***

The compound **1** (1.14 g, 4.8 mmol) in 1,4-dioxane (40 mL) was treated with $Na_2S.9H_2O$ (5.16 g, 20.8 mmol) for 4 h at 90 °C till completion (TLC). After cooling the reaction mixture, HCl (4 ml) and $NaHCO_3$ (1.00 g) were added to adjust the pH = 8-9. The yellow precipitates obtained were filtered to obtain crude **2**, which were further re-crystallized from ethanol-water mixture and dried under vacuum to obtain analytically pure yellow solid **2** (78.6%). IR (KBr) ν_{max} : 1171, 1301, 1498, 1517, 1573, 1596, 1606, 1625, 1892, 2206, 3317, 3385 cm^{-1}. ^{1}H NMR (500 MHz,

$CDCl_3$ + DMSO-d_6, 25 °C) δ (ppm): 4.24 (s, 2H, -NH_2), 6.19 (d, *J* = 10 Hz, 2H, ArH), 6.36 (d, *J* =5 Hz, 2H, ArH), 6.80 (s, 1H, -CH), 6.86 (d, *J* =5 Hz, 2H, ArH), 7.21 (d, *J* = 10 Hz, 2H, ArH) and 9.19 (s, 1H, -OH). ^{13}C NMR (125 MHz, $CDCl_3$ + DMSO-d_6, 25 °C) δ (ppm): 112.35, 119.46, 120.70, 123.81, 128.11, 130.41, 131.41, 135.52, 142.77, 153.39, 164.04. HRMS: *m/z* calculated for $C_{15}H_{12}N_2O$: 237.1022 and found: 237.1045 (M^++1).

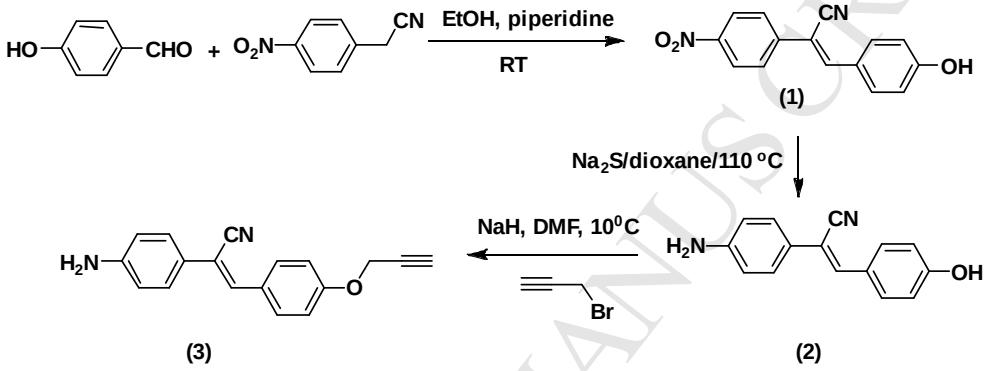
*2.6.3. Synthesis of (Z)-2-(4-aminophenyl)-3-(4-(prop-2-ynyloxy)phenyl)acrylonitrile **3***

To the suspension of NaH (0.05 g, 2.0 mmol) in DMF at 10 °C was added solution of **2** (0.5 g, 2.1 mmol) in 10 ml DMF and stirred for 30 min at same temperature. Propargyl bromide (0.5 g, 4.2 mmol) was added dropwise to the reaction mixture and stirred for 2 h for completion (TLC). The reaction mixture was then diluted with cold water and extracted with EtOAc. The organic layer was dried over anhydrous Na_2SO_4 and solvent was removed under reduced pressure to obtain crude **3**, which was further purified by column chromatography using neutral alumina and EtOAc:hexane (10:90) as eluents to yield pure **3** (63%). IR (KBr) ν_{max} : 1021, 1178, 1247, 1378, 1505, 1508, 1625, 2127, 2212, 2917, 3266, 3355, 3436 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$, 25 °C) δ (ppm): 2.55 (t, *J* = 2.5 Hz, 1H, -CH), 3.86 (s, 1H, -NH_2), 4.74 (d, *J* =2 Hz, 2H, -CH_2), 6.71 (d, *J* =10 Hz, 2H, ArH), 7.03 (d, *J* =5 Hz, 2H, ArH), 7.30 (s, 1H, -CH), 7.45 (d, *J* =5 Hz, 2H, ArH) and 7.83 (d, *J* =10 Hz, 2H, ArH). ^{13}C NMR (125 MHz, $CDCl_3$, 25 °C) δ (ppm): 56.04, 76.10, 78.23, 109.71, 114.96, 115.29, 115.37, 118.79, 125.06, 127.21, 128.04, 130.21, 130.77, 131.49, 138.16, 141.56, 147.41, 158.83. HRMS: *m/z* calculated for $C_{18}H_{14}N_2O$: 274.1111 and found: 274.1125 (M).

3. Results and Discussion

3.1 Synthesis and characterization

The probe **3** was readily synthesized from the propargylation of the hydroxyl group of **2** with 3-bromo-1-propyne in DMF with 63% yield (Scheme 1). All compounds exhibited satisfactory spectroscopic and analytical data. (Fig. S1-S10).



Scheme 1: Synthetic route to probe **3**.

3.2. pH studies

As we know that pH sensitivity of a sensing probe is of importance for practical applications in both environmental and biological analysis. Therefore, before evaluating the metal ion binding properties of 3, the acid-base titration experiment was performed which revealed the stability of 3 over a wide pH range (5-11), thus qualifying for the use without interference from pH effects in biological samples (Fig. S12).

3.3. Cation sensing studies

The emission spectrum of **3** (1 x 10^{-5} M, in EtOH:HB, 10:90 *v/v* mixture, pH 7.4) (HB=HEPES buffer, 0.1 M containing 0.1 M NaOH, pH 7.4) is characterized by a strong emission band at 517

nm (Φ_f = 0.130) when excited at 351 nm. We attribute this emission behaviour of **3** to the aggregation through intermolecular π-π interactions as the stilbene derivatives with in-built elastic behaviour are known for their ability to exhibit aggregation induced emission [40]. The behaviour of **3** towards different metal ions was investigated. As depicted in Fig. S13, the emission intensity remains unperturbed in the presence of Hg^{2+}, Fe^{3+}, Cu^{2+}, Ni^{2+}, Zn^{2+}, Mn^{2+}, Mg^{2+}, Fe^{2+}, Pb^{2+}, Na^{2+}, Ba^{2+}, K^{+}, Co^{2+}, Al^{3+}, Cd^{2+}, Gd^{3+}, Pr^{3+}, Ce^{3+}, Sm^{3+}, Tb^{3+}, Nd^{3+}, La^{3+}(added up to 3.3 x 10^{-4} M) except Pd^{2+} ions which resulted in the quenching of emission under similar experimental conditions. Additionally, in the presence of anions also, such as OH^-, Cl^-, Br^-, I^-, $H_2PO_4^-$, CN^-, CH_3COO^-, PO_4^{3-}, HSO_3^-, HSO_4^-, NO_3^-, $C_6H_5O_7^{3-}$, CO_3^{2-} (added upto 3.3 x 10^{-4} M), no change in the emission intensity was noticed (Fig. S14). These results indicate the high selectivity of **3** for the Pd^{2+} ions.

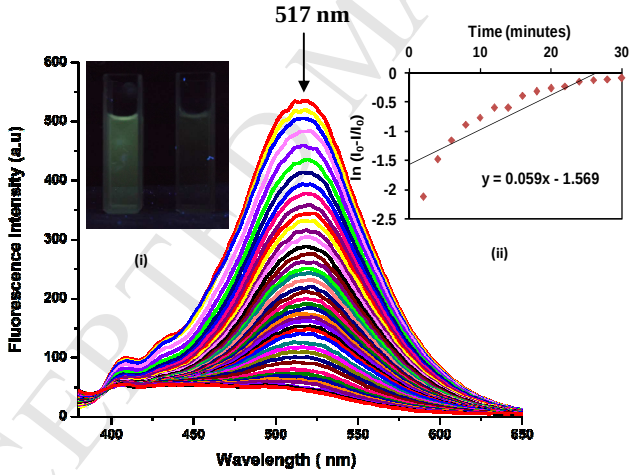


Fig. 1. Changes in emission spectra of **3** (1 x 10^{-5} M, in EtOH:HB, (10:90 *v/v* mixture, pH 7.4 at $\lambda_{exc.}$= 351nm) upon incremental additions of Pd^{2+} solution (1 x 10^{-5} to 9 x 10^{-5} M). Inset: (i) Visual change in emission of **3** upon addition of Pd^{2+} under illumination of 365nm), (ii) emission behaviour of **3,** λ_{em} = **517 nm** with time at 7.5 x 10^{-5} M solution of Pd^{2+}.

In order to quantify the results for the detection of Pd^{2+} ions, $PdCl_2$ solution was used over other palladium species owing to its high toxicity among other species. Upon gradual addition of 15mM solution of Pd^{2+} ions (1 x 10^{-5} to 9 x 10^{-5} M)to the solution of **3** (1 x 10^{-5} M, in EtOH:HB, 10:90 *v/v* mixture, pH 7.4), the emission intensity of **3** showed a gradual decrease as shown in Fig. 1, attaining saturation when addition of 7.5 x 10^{-5} M was achieved, and the process was completed only after 30 min. with rate constant K = 0.13 s^{-1} (Fig. 1, Inset). However, when the lower concentrations (5 x 10^{-5} M and 2.5 x 10^{-5} M) was employed, a similar change could be achieved much slower in 66 min. (K = 0.06 s^{-1}) and 96 min. (K = 0.04 s^{-1}) respectively, (Fig. S15). We propose the sensing protocol as the palladium ions promoted hydrolysis reaction releasing free hydroxyl derivative **2** in the solution (Fig. 2).

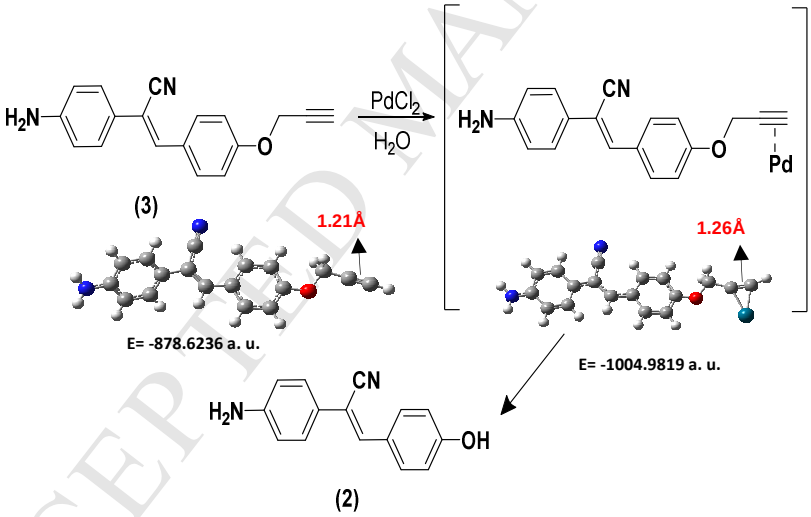


Fig. 2. Possible mechanism for the detection of palladium using the optimized structures of **3** and the intermediate **3**:Pd^{2+} complex (using B3LYP/6-31G and B3LYP/LanL2DZ, Gaussian 09, respectively) [39].

In order to substantiate the sensing protocol, we recorded the change in the particle size after the addition of Pd^{2+} ions to the solution of **3** using dynamic light scattering (DLS) studies. The

experimentally found small particle size (43.82 nm), obtained after the addition of Pd^{2+} ions to the solution of **3** (particle size 458.7 nm) (Fig. 3a,b) suggested that *in-situ* released compound **2** was no longer in the crystalline aggregated state. This change in the physical state is further supported by the transmission electron micrographs (TEM) and electron diffraction (ED) pattern (Fig. 3c-f). The non-emissive nature of the solution with *in-situ* formed **2** may be attributed to the intramolecular motions in the isolated (disaggregated) cyano-stilbene based compounds, responsible for non-radiative relaxation [40,41]. The proposed mechanism through the formation of **2** was further confirmed from the HRMS spectrum. The peak at *m/z* 237.0992 [M^{+}+1] corresponding to compound **2**, was observed in the HRMS spectrum of the product mixture (Fig. S11), in addition to comparison using thin layer chromatography.

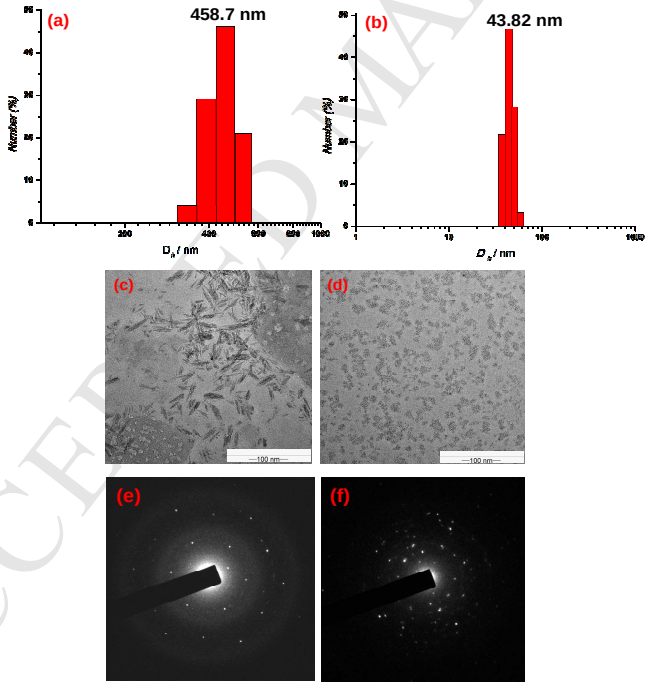


Fig. 3. DLS graph (a) **3** and (b) **3** + Pd^{2+}; TEM images (c) solution of **3** and (d) solution of **3** + Pd^{2+}; ED pattern (e) **3** and (f) **3** + Pd^{2+} (in EtOH:HB, 10:90 *v/v* mixture, pH 7.4).

On the basis of the changes in fluorescence intensity, the detection limit for Pd^{2+} was calculated to be 8.8 x 10^{-8} M with palladium content of 9.3 ppb, which is drastically lower than the permissible level of palladium ions (5-10 ppm) by the European Agency for the Evaluation of Medicinal Products (EMEA) [11] and moreover, is the significantly lower limit of detection reported for the detection of palladium ions *via* the catalyzed hydrolysis reaction (Table 2). Further it could be noticed in the Fig. 4 that **3** responds selectively to Pd^{2+} ions even in the presence of other relevant competing metal ions in neutral aqueous medium (For anions, Fig. S16). However, owing to instability, interference from Au^{2+} ions could not be checked.

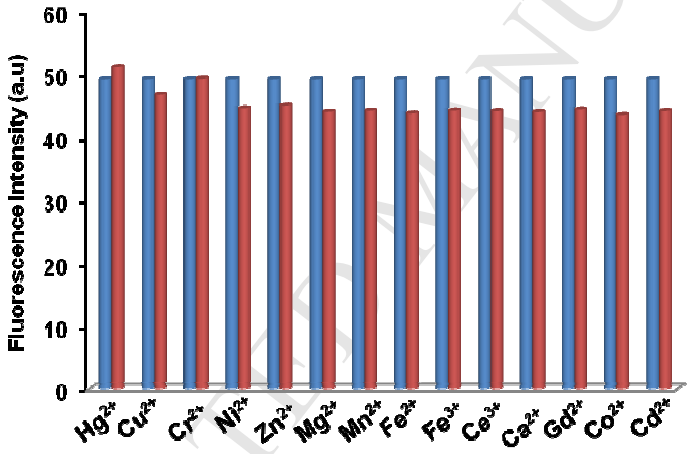


Fig. 4. Changes in emission behaviour of **3** (1 x 10^{-5} M, in EtOH:HB , 10:90 *v/v* mixture, pH 7.4, $\lambda_{exc.}$= 351nm) upon addition of various cations (upto 3.3 x 10^{-4} M) in the presence of Pd^{2+} ion solution (7.5 x 10^{-5} M).

The UV-visible absorption spectrum of **3** (1 x 10^{-5} M, in EtOH:HB, 10:90 *v/v* mixture, pH 7.4) displays a moderate intensity absorption band at 351 nm and a weak absorption in the range of 270-320 nm (Fig. S17). However, the gradual addition of the solution of Pd^{2+} ions (0-7.5 equiv.) did not cause any significant change except a slight bathochromic shift in the main absorption

band and increased absorption in the 270-320 nm range, which indeed is the indication of the formation of different chemical species in solution in the presence of Pd^{2+} ions.

4. Practical application

As pointed out in section 1, urine is one of the major routes for the excretion of Pd^{2+} ions, with the Pd^{2+} level in the range 0.006 to 0.3 μg/L in the adult human urine sample. [38] Thus the applicability of **3** to detect Pd^{2+} in the real sample was also evaluated. For that a calibration curve (Fig. 5), was generated by the successive addition of Pd^{2+} ions (0.5-4 equiv.) to the solution of **3** (1 x 10^{-5} M, in EtOH:HB, 10:90 *v/v* mixture, pH 7.4).

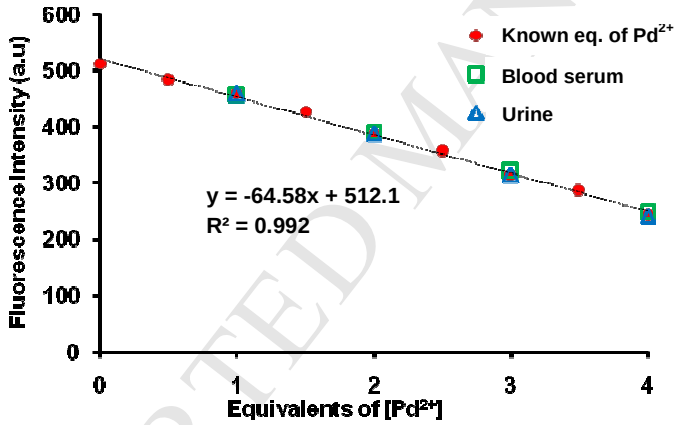


Fig. 5. Changes in the emission behaviour of **3** (1 x 10^{-5} M, in EtOH:HB, 10:90 *v/v* mixture, pH 7.4, $\lambda_{exc.}$= 351 nm) in the presence of known equiv. of Pd^{2+} as well as urine and blood serum samples spiked with known equiv. of Pd^{2+}.

A human urine sample obtained from a clinical laboratory, was diluted 100 times with MeOH:H_2O, 1:1 *v/v* mixture followed by filtration to remove any suspended particles. For monitoring the emission changes, when 20 µl of the diluted urine sample was added to the 3 ml solution of **3**, negligible change was observed in the emission intensity. Then, the solution of **3** +

urine was spiked with 1, 2, 3 and 4 equiv. of Pd^{2+} ions (15 mM) and emission changes were recorded (marked as green in Fig. 5). Similar experiment was performed for the detection of Pd^{2+} in human blood serum obtained from a health centre and diluted with distilled water (marked as blue in Fig. 5). The noted significant % age of spiked recoveries in both cases (Table 1) depict promising potential of **3** for practical application.

Table 1. Detection of Pd^{2+} in biological fluids.

Urine			**Human Blood Serum**		
No. of equivalents of Pd^{2+}		**Recovery ± SD (%)**	**No. of equivalents of Pd^{2+}**		**Recovery ± SD (%)**
Added	**Found[a]**		**Added**	**Found[a]**	
0	0	-	0	0	-
1	0.85	85.0±0.29	1	0.83	83.9±0.26
2	1.93	96.5±0.11	2	1.91	95.5±0.37
3	3.07	102.3±0.13	3	2.96	98.66±0.15
4	4.19	104.7±0.05	4	4.10	102.5±0.15

[a] Mean of three readings. SD- standard deviation

Conclusions

In conclusion, we have synthesized a propargylated cyano-stilbene probe, which detects Pd^{2+} ions with significantly lower limit of detection (9.3 ppb) under mild conditions, in comparison to the contemporary probes employing the similar mechanism (Table 2). Based upon the Pd^{2+} catalyzed depropargylation reaction, free hydroxyl moiety is released in the solution. Importantly, the reported sensing protocol is first time employed for the detection of Pd^{2+} ions from the human urine sample besides its application in human blood serum.

Table 2. Literature reports on the detection of Pd^{2+} employing Pd^{2+} catalysed depropargylation reaction.

Solvent	Oxidation states	Reaction time (h)	Detection limit	Application	Ref.
10:90 $CH_3CN:H_2O$	Pd^0, Pd^{2+}, Pd^{4+}	1	0.03 µM (30 nM)	Cell study in Zebrafish	35
PBS (20 mM)	Pd^0, Pd^{2+}, Pd^{4+}	2	0.07 µM (70 nM)	live RAW 264.7 macrophage cells study	34
20:80 $CH_3CN:H_2O$ (10mM HB)	Pd^0, Pd^{2+}, Pd^{4+}	3	87 nM	No application	33
80:20 EtOH:PBS	Pd^0, Pd^{2+}, Pd^{4+}	0.5	0.05 µM (50 nM)	No application	36
70:30 $DMSO:H_2O$	Pd^0, Pd^{2+}, Pd^{4+}	0.5	340 nM	Live HeLa cells study	32
50:50 EtOH:PBS	Pd^{2+}	-	93 nM	Living cell study	31
10:90 EtOH:HB	Pd^{2+}	0.5	9.3 ppb (88 nM)	Urine and human blood serum sample	**This work**

Acknowledgements

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Supporting Information

Spectral data, Fluorescence, UV-visible, detection limit calculations and complete reference of Gaussian09 [39].

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Highlights:

- The probe detects Pd^{2+} ions in significantly low limit of detection of 9.3 ppb.
- Sensing protocol utilizes the potential of Pd^{2+} to catalyse a chemical reaction.
- Depicts significant %age of spiked recoveries of Pd^{2+} in human urine and blood serum.
- Works under mild conditions in comparison to other contemporary probes.