A bio-compatible pyridine-pyrazole hydrazide based compartmental receptor for Al³⁺ sensing and its application in cell imaging

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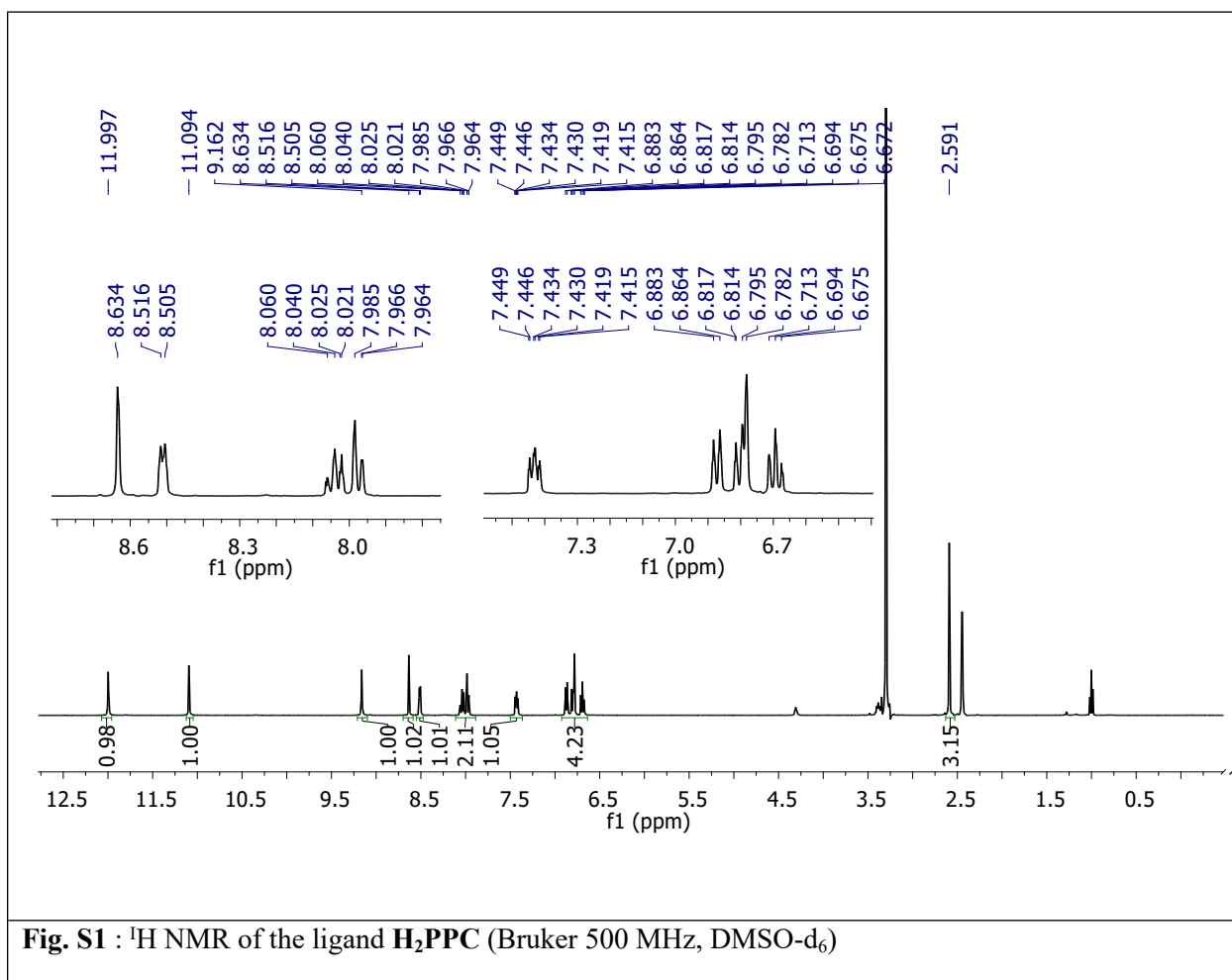
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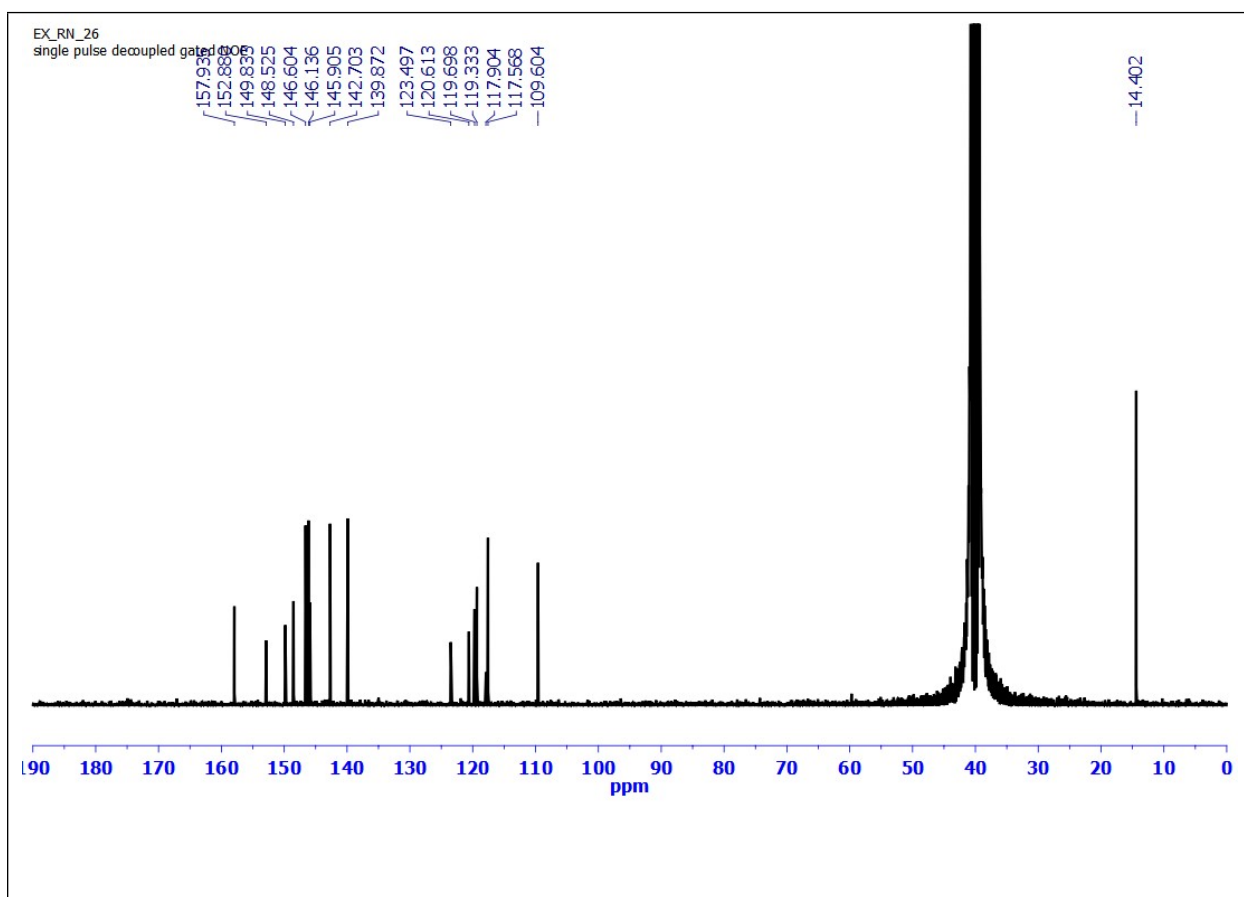
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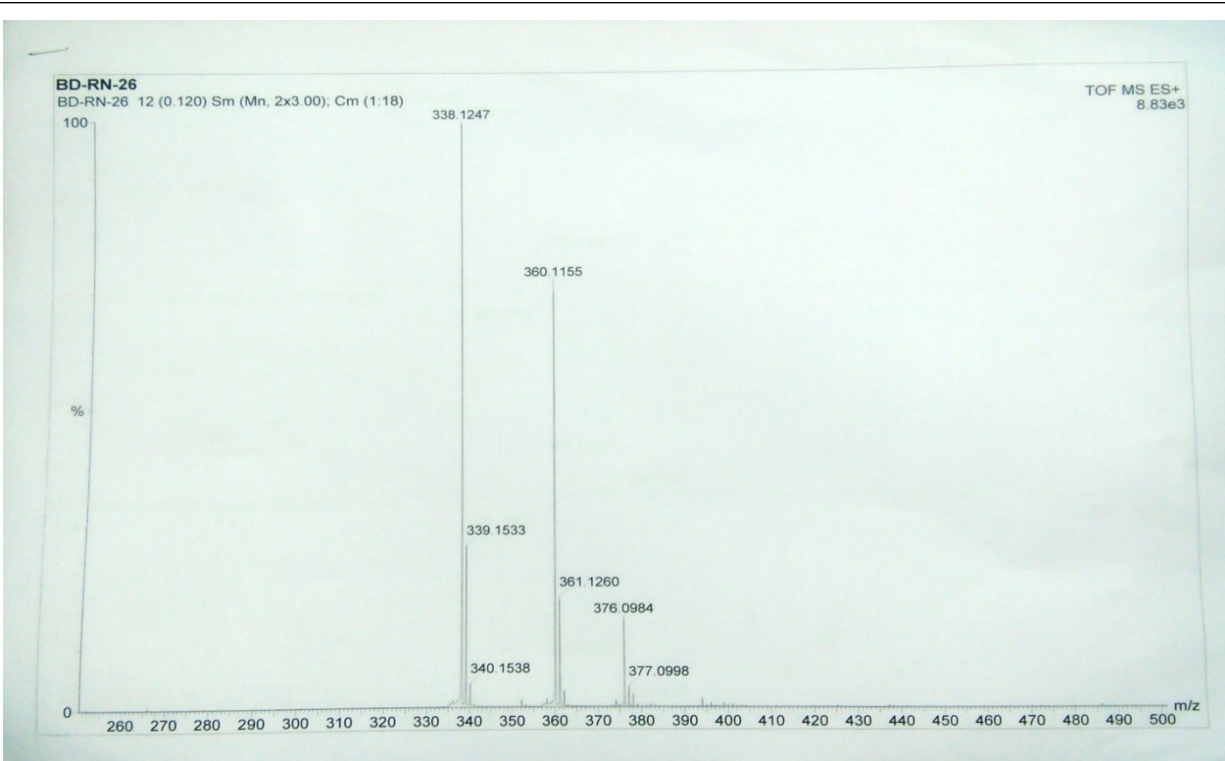[h]*Department of Chemistry, Mahishadal Raj College, Purba Medinipur, 721628, India*

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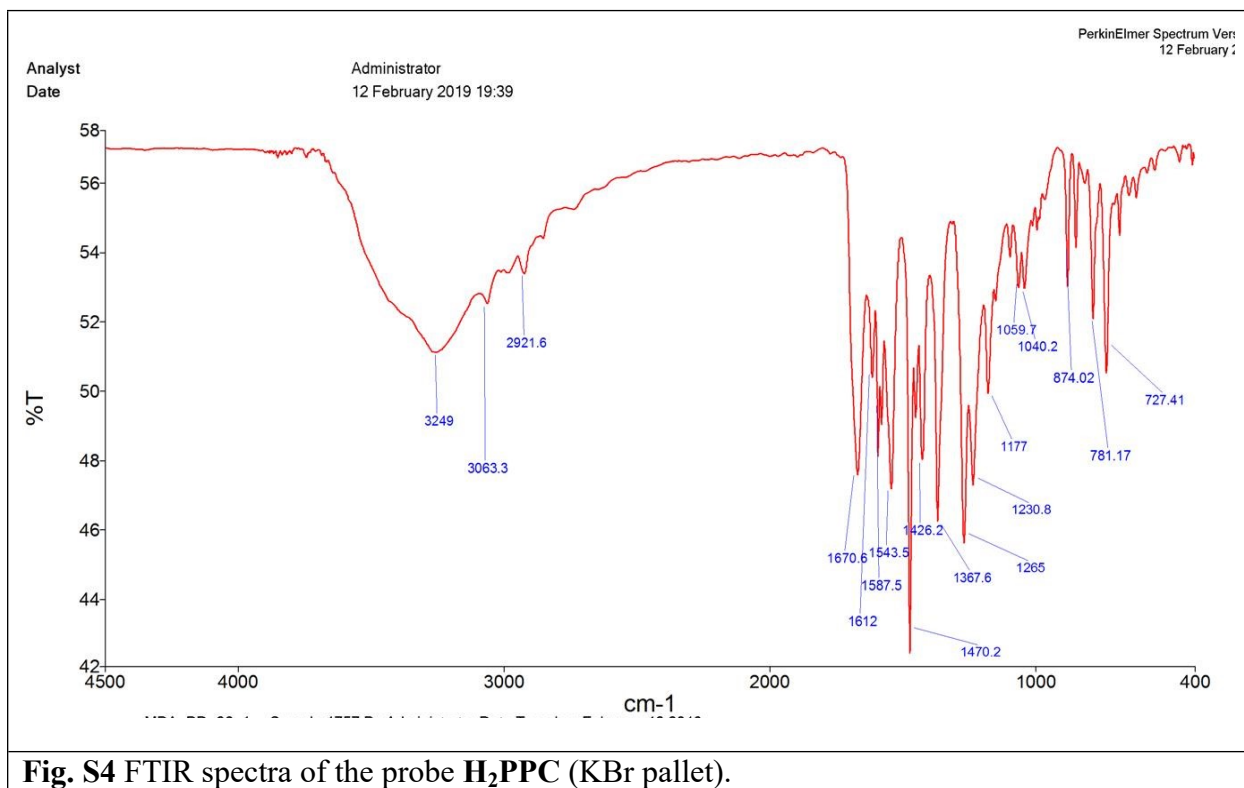**Fig. S1** : ¹H NMR of the ligand **H₂PPC** (Bruker 500 MHz, DMSO-d₆)

EX_RN_26
single pulse decoupled gated NOE

157.935
152.880
149.839
148.525
146.604
146.136
145.905
142.703
139.872
123.497
120.613
119.698
119.333
117.904
117.568
109.604
14.402

Fig. S2 : ^{13}C NMR of the ligand **H$_2$PPC** (Bruker 125 MHz, DMSO-d$_6$)

Fig. S3 Mass spectra of ligand **H₂PPC**. 337.1175 (calculated), 338.1247 (observed) correspond to (**H₂PPC** +H⁺), and 360.1155 correspond to (**H₂PPC** +Na⁺)

Fig. S4 FTIR spectra of the probe **H₂PPC** (KBr pallet).
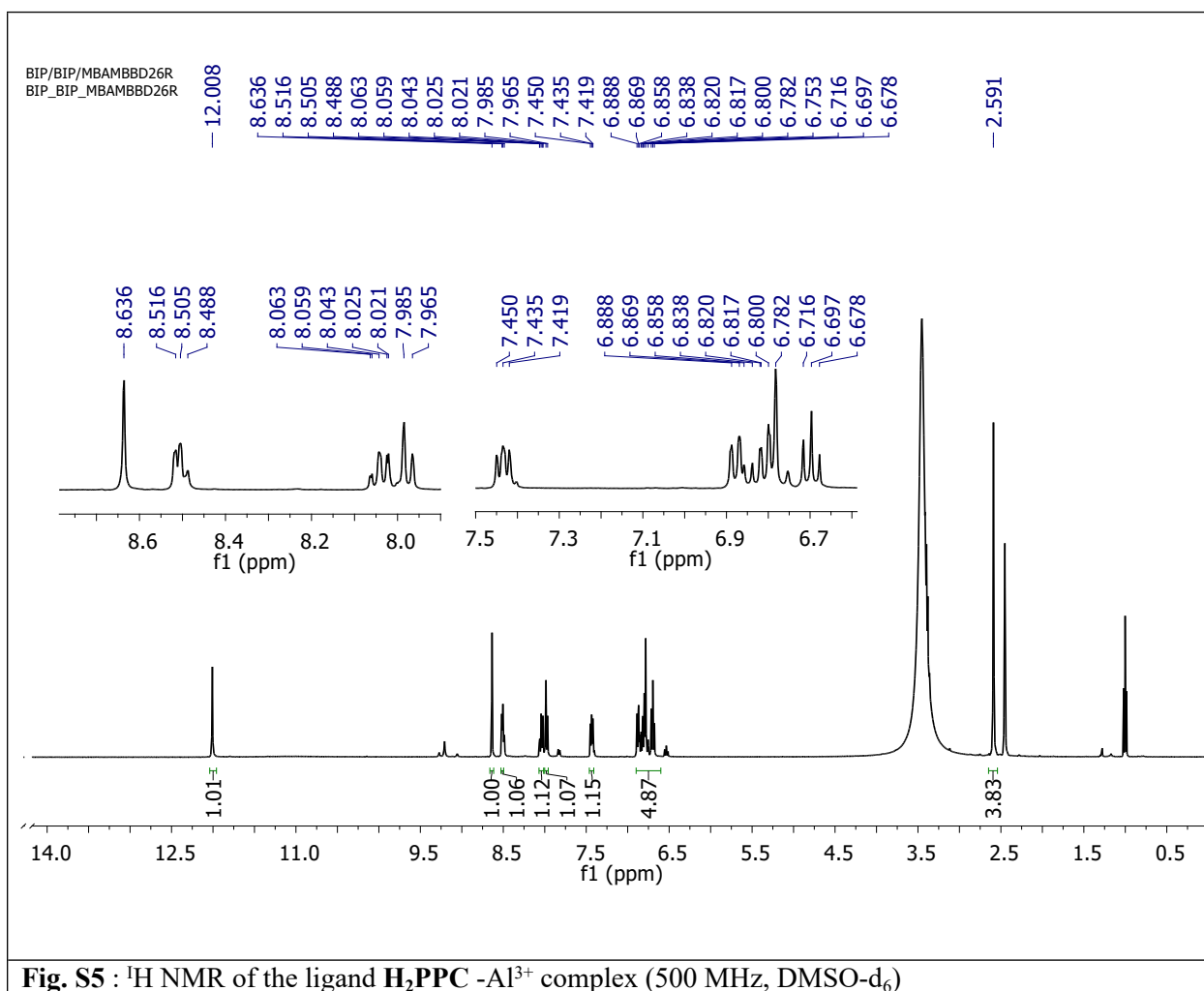
Fig. S5 : ^{1}H NMR of the ligand **H$_2$PPC** -Al^{3+} complex (500 MHz, DMSO-d$_6$)

EX_RN_26 Al

158.108
152.760
150.015
148.521
146.551
146.023
142.782
139.941
123.614
123.600
121.282
120.770
119.809
117.949
117.648
109.533
14.302

Fig. S6 : ^{13}C NMR of the ligand **H$_2$PPC-Al^{3+}** complex (Bruker 125 MHz, DMSO-d$_6$)

Fig. S7 Mass spectra of the **H₂PPC** + Al³⁺ complex (2:1). Calculated 699.2009, observed 699.1948 correspond to [2**H₂PPC**+ Al³⁺- 2H⁺]
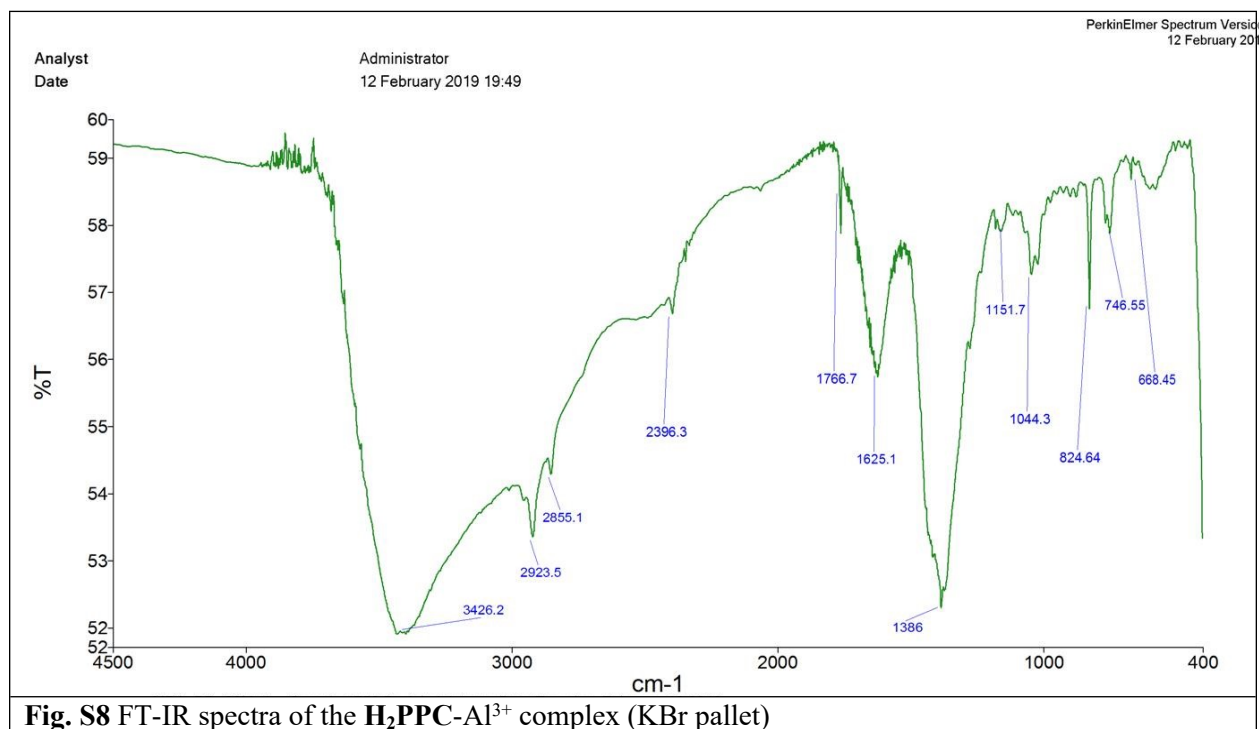
Fig. S8 FT-IR spectra of the **H₂PPC**-Al³⁺ complex (KBr pallet)
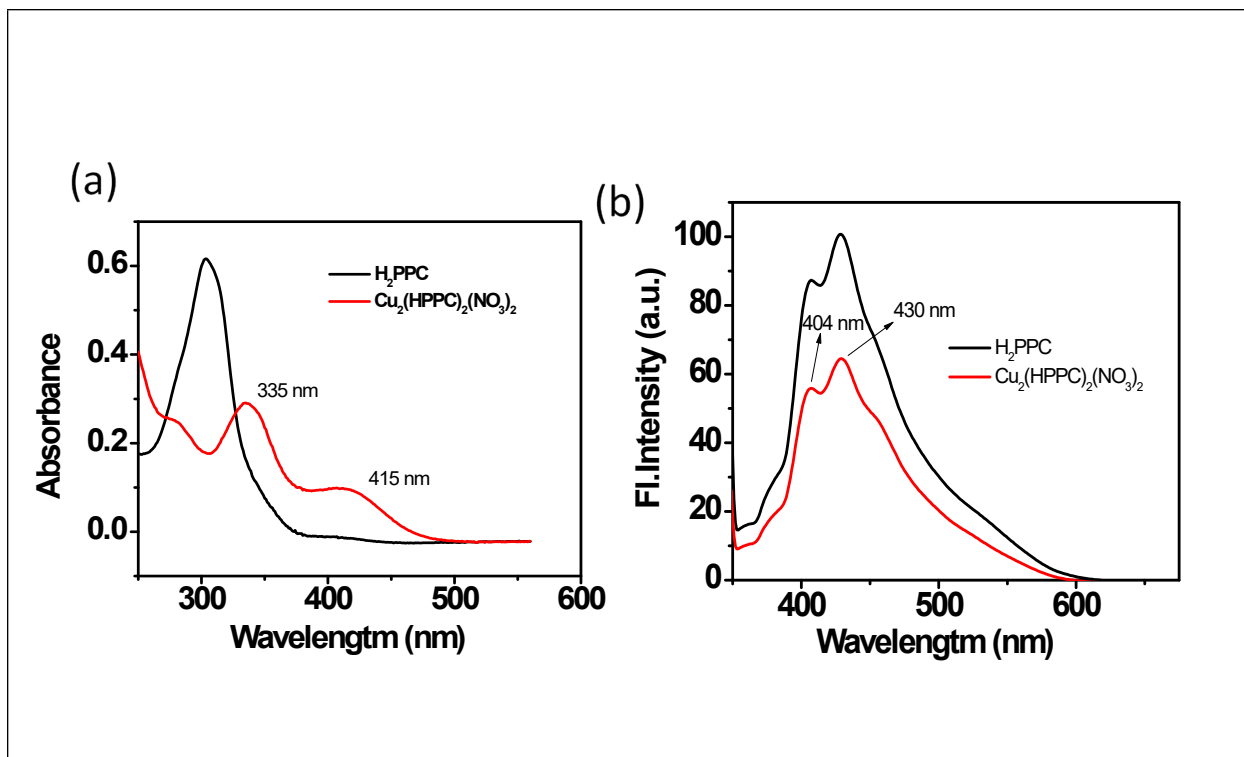
Fig. S9 : (a) absorption spectra and (b) emission spectra (λ_{ex} = 340 nm) of **H₂PPC** and **H₂PPC-Cu²⁺** complex in MeOH-H$_2$O (9:1,v/v)
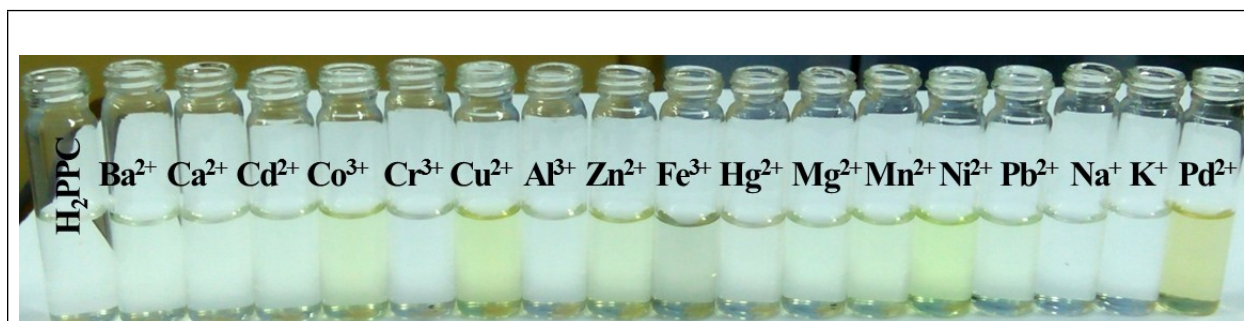
Fig. S10 photographic image of **H₂PPC** + different metal ions under ambient light in MeOH-H$_2$O (9:1,v/v)
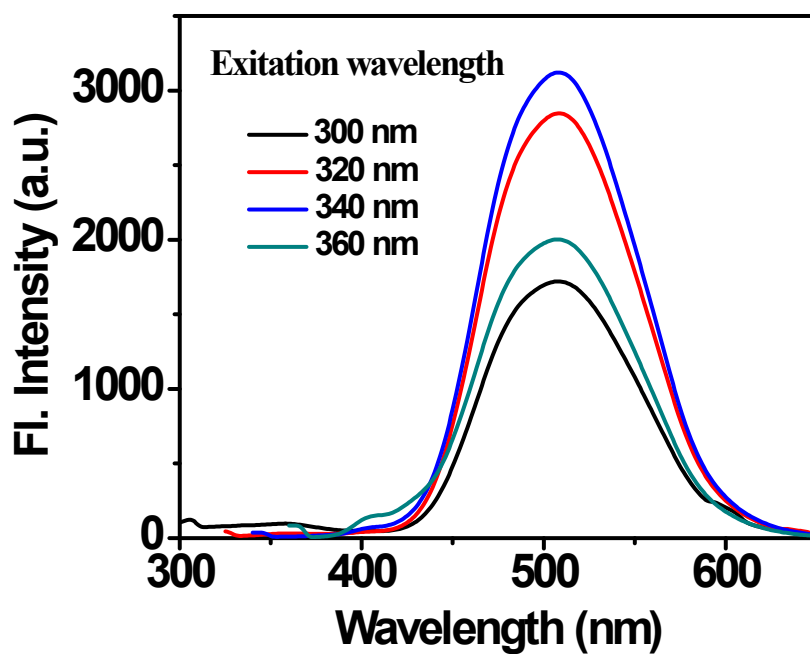
Fig. S11 Graph for excitation wavelength variation of **H₂PPC** with Al^{3+} in MeOH-H$_2$O (9:1,v/v)
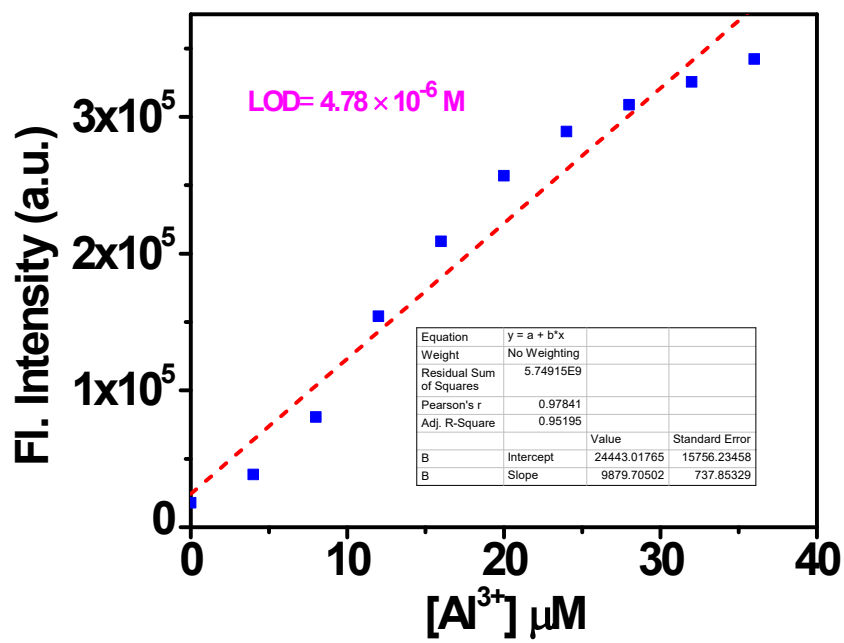
Fig. S12 plot for calculation of detection limit

Fig. S13 (a) Absorption and (b) emission spectra of **H₂PPC** and **H₂PPC** with Al^{3+} in different organic solvent (inset: corresponding image under UV-light, λ = 365 nm) (c) **H₂PPC**+ Al^{3+} in different water content of methanol under UV-light, λ = 365 nm.
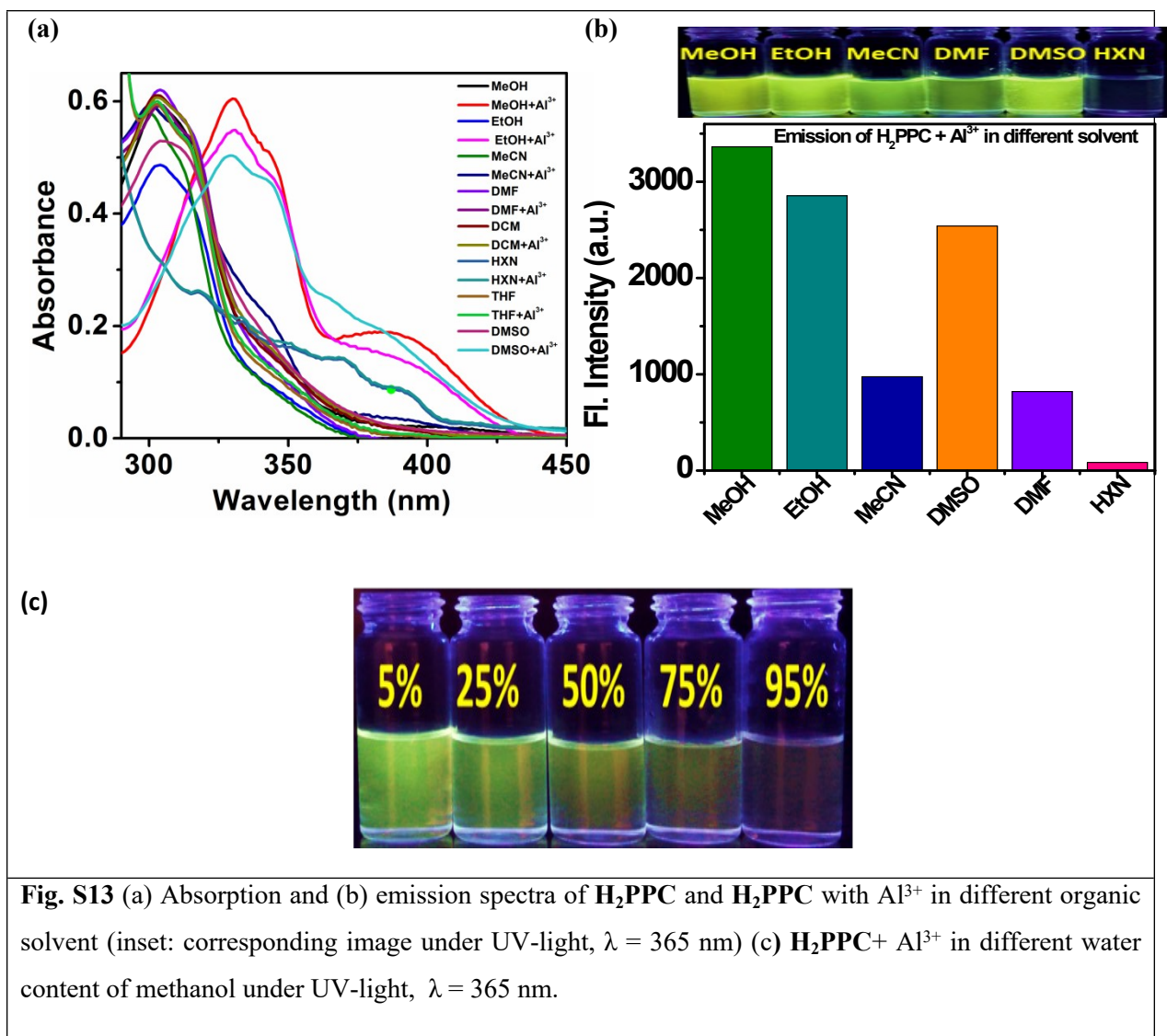
Fig. S14 Jobs' plot from fluorescence titration results

Table-S1

Crystallographic data of **H₂PPC** and **2**

	C₁₇H₁₅N₅O₃ (**H₂PPC**)	C₃₄H₂₈Cu₂N₁₂O₁₂ (**2**)
Empirical formula	$C_{17}H_{15}N_5O_3$ (**H₂PPC**)	$C_{34}H_{28}Cu_2N_{12}O_{12}$ (**2**)
Formula weight	337.34	923.78
Crystal system	Tetragonal	Triclinic
Space group	I41/a (No. 88)	P-1 (No. 2)
a/ Å	19.559(2)	8.4663(13)
b/ Å	19.559(2)	9.2044(14)
c/ Å	16.966(3)	11.8424(18)
α/°	90	73.774(2)
β/°	90	70.534(2)
γ/°	90	80.563(2)
V/A³	6490.4(15)	832.9(2)
Z	16	1
Density(D/gcm⁻³)	1.381	1.842
μ/mm⁻¹	0.098	1.368
F(000)	2816	470
Temperature(K)	150	100
Θ range for data collection/⁰	1.6, 25.9	1.9, 28.0
Dataset	-23: 19 ; -23: 23 ; -20: 20	-9: 11 ; -11: 11 ; -11: 15
Tot., Uniq. Data, R(int)	14751, 2939, 0.038	9623, 3719, 0.046
Observed data [I > 2.0 σ(I)]	1798	3071
Nref, Npar	2939, 239	3719, 280
R, wR2, S	0.0419, 0.1150, 1.01	0.0578, 0.1653, 1.10

Table-S2

Selected bond distances (Å) and angles (°) data for **2**

Selected Bonds	Value(Å)	Selected Angles	(°)
Cu1-O3	1.897(2)	O3-Cu1-O4	91.58(12)
Cu1-O4	2.357(3)	O3-Cu1-N1	98.08(13)
Cu1-N1	2.053(4)	O3-Cu1-N3	172.53(14)
Cu1-N3	1.919(3)	O3-Cu1-N5	95.48(13)
Cu1-N5	2.010(4)	O3-Cu1-O3#	84.69(11)
Cu1-O3#	2.730(3)	O4-Cu1-N1	97.53(13)
		O4-Cu1-N3	95.42(14)
		O4-Cu1-N5	83.01(13)
		O3#-Cu1-O4	171.62(9)
		N1-Cu1-N3	78.45(14)
		N1-Cu1-N5	166.41(13)
		O3#-Cu1-N1	90.43(12)
		N3-Cu1-N5	87.97(15)
		O3#-Cu1-N3	88.70(12)
		O3#-Cu1-N5	89.86(12)

		Cu1-O3-Cu1#	95.31(12)

Symmetry #=1-x,1-y,-z

Table-S3

Details of hydrogen bond distances (Å) and angles (°) of **H₂PPC** and complex-**2**

Compound	D-H...A	d(D-H)	d(H...A)	d(D...A)	<(DHA)
H₂PPC	N4-H4N ···O1	0.87(2)	2.167(19)	2.963(2)	152.6(16)
	O2-H2 ···N5	0.99(3)	1.73(3)	2.600(2)	145(3)
Complex-2	N4-H4N ···O6	0.75(6)	2.32(6)	2.969(6)	145(6)
	O2-H2O ···O5	0.85(6)	2.15(6)	2.774(5)	129(6)

Table S4: Ionic radii and ionic potential of relevant cations

Cations	Ionic radius [a] (Å)	Ionic potential (Z/r)	Hydrated radius [b] (Å)	Ionic potential (z/r)
Na^+	0.95	1.05	3.58	0.279
K^+	1.33	0.75	3.31	0.302
Ba^{2+}	1.35	1.48	4.04	0.495
Ca^{2+}	0.99	2.02	4.12	0.485
Cd^{2+}	0.97	2.06	4.26	0.469
Co^{2+}	0.72	2.63	4.23	0.472
Cr^{3+}	0.64	4.68	4.61	0.650
Cu^{2+}	0.72	2.78	4.19	0.477
Zn^{2+}	0.74	2.70	4.30	0.465
Al^{3+}	0.50	6	4.75	0.631
Fe^{3+}	0.60	5	4.57	0.656
Hg^{2+}	1.02	1.96	-	-
Mg^{2+}	0.65	3.07	4.28	0.467
Mn^{2+}	0.80	2.50	4.38	0.456
Ni^{2+}	0.70	2.85	4.04	0.495
Pb^{2+}	1.32	1.51	4.01	0.498
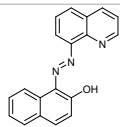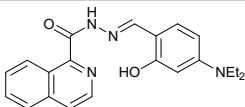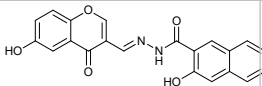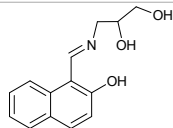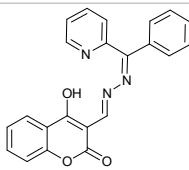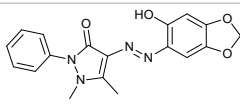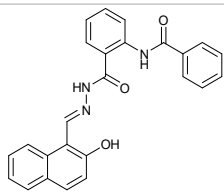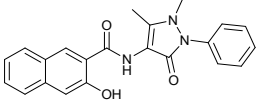
a L. Pauli "Nature of the Chemical Bond," Cornell University Press, 2nd. ed., Ithaca, N. Y., **194,**
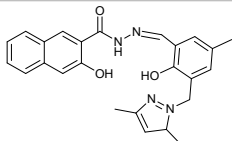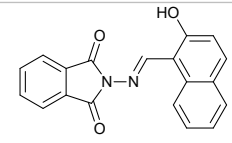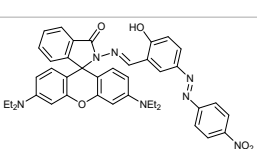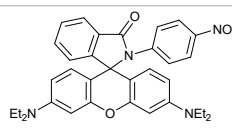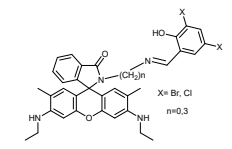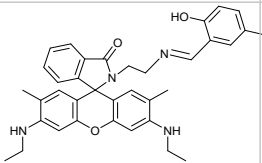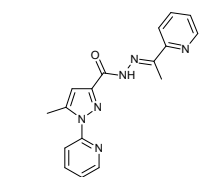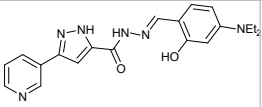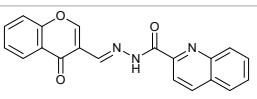
b E.R. Nightingale, Phenomenological theory of ion solvation. effective radii of hydrated ions, ***J. Phys. Chem. C*** 63 (1959) 1381–1387
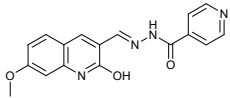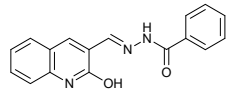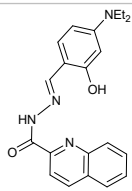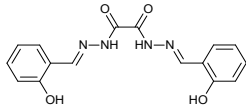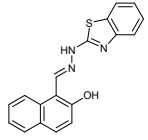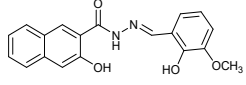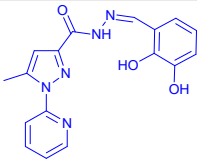
Table S5: Selective bond distance and bond angles of **1 from DFT**

Bond	Bond distance(Å)	Bond	Bond angles(°)
Al1-O1	1.865	N1-Al1-O1	87.23
Al1-O2	1.890	O1-Al1-O2	89.82
Al1-N1	1.971	N1-Al1-O2	80.92
Al1-N2	1.929	N1- Al1-N2	105.13
Al1-N3	1.999	O1- Al1-N2	163.75
Al1-N4	1.951	O2- Al1-N2	81.95
		N3- Al1-N4	103.82
		O1- Al1-N3	80.93
		O1- Al1-N4	93.53
		O2- Al1-N3	87.14
		O2- Al1-N4	168.91

Table S6: some recent published work on Al^{3+} sensor

Sl	Sensor Structure	Excitation and emissiom maxima (nm)	Medium	Detection limit	Interference	Paper strip based sensing	imaging in Live Cell	Reference
1		537, 612	H$_2$O	0.69 Nm	No cations, F-	yes	no	[1]
2		467, 545	MeOH	0.5 Nm	Cu^{2+}, Co^{2+}, Ni^{2+}, Fe^{2+}	no	MCF-7	[2]
3		435, 503	EtOH: H$_2$O (3: 1, v/v)	0.348 Nm	Cu^{2+}, Ni^{2+}, Fe^{3+}	yes	NO	[3]
4		307, 436	MeOH:H$_2$O (2:8, v/v)	45 µM	NO	no	HepG2 cell	[4]
5		380, 481	MeOH:H$_2$O (1:1, v/v)	3.91Nm	Co^{2+}, Ni^{2+}	yes	AGS cells	[5]
6		450, 613	MeOH: H$_2$O	35 µM	no	yes	no	[6]
7		430, 480	MeOH: H$_2$O (8:2)	1.68 Nm	no	yes	MDA-MB-468	[7]
8		360, 505	MeOH	28 nm	Fe^{3+}	no	no	[8]

9		487, 493	MeOH-H$_2$O (4:6)	114 nM	no	yes	HepG2	[9]
10		375, 472	DMSO-H$_2$O (1:9 **v/v**)	1 ppb	nd	yes	no	[10]
11		500, 582	EtOH-H$_2$O (4:1)	11 μM	no	NO	NO	[11]
12		490, 578	MeCN/H$_2$O 1:8	0.3 μM	no	yes	zebrafish brain tissue	[12]
13		345, 555	MeOH-H$_2$O (1:9)	1.4-5.3 nM	no	NO	MDA-MB-468	[13]
14		500, 552	MeOH-H$_2$O (9:1)	2.86 nM.	Cu^{2+}, Fe^{3+}, Ni^{2+} and Co^{2+}	No	E. coli, B.s ubtilis C. albican E. histolytica	[14]
15		300, 450	DMSO-H$_2$O (2:8)	1.2 nM	no	yes	HepG2	[15]
16		400, 468	H$_2$O, HEPES	0.062 μM	Cu^{2+}	no	HeLa cell	[16]
17		435, 520	MeOH-H$_2$O (7:3,v/)	7.6 nM	F-	no	Verocells	[17]

No.	Structure	λ (nm)	Medium	LOD	Analyte	Cell permeable	Cell	Ref.
18		380, 443	water	56 nM	PPi	no	MCF-7	[18]
19		390, 442	HEPES buffer	0.72 nM	Cu^{2+} and Fe^{3+}	Yes	no	[19]
20		390, 440 and 480 590 ,	MeCN-H_2O (1:4)	0.104, 4.17μM	no	no	HeLa cells	[20]
21		410, 491	bis-tris buffer	2.01 μM	Cu^{2+}, Fe^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+} and Cr^{3+}	no	HeLa cells	[21]
22		420, 515	DMSO-H_2O (7:3)	ND	Fe^{2+}, Fe^{3+}, Cu^{2+}	NO	NO	[22]
23		330, 524	MeOH-HEPES buffer (9:1)	1.7 μM	Cu^{2+}	YES	no	[23]
24	**H₂PPC**	340, 506	MeOH-HEPES buffer (9:1)	4.78 μM	Cu^{2+}, Fe^{3+}, $H_2PO_4^-$	yes	Vero cell, A549 cells	This work

Details of other spectroscopic and related biological measurement and analysis:

Details of general method for UV-Vis and PL studies, Detection limit calculation [24] , Fluorescence quantum yield calculation [24], Fluorescence lifetime measurements [25–27], Computational details [28–37], Cell Culture and Imaging Study [38–40] and Cytotoxicity assay [41] were performed according to literature. The related description incorporated in the supporting information part, Page S20-S23, **ESI**).

2.3 General method for absorbance and emission measurements

The stock solution of chemosensor **H₂PPC**(1.0 mM) was prepared in methanol. The solution of **H₂PPC** was then diluted to 20 μM with methanol. Solutions of the guest metal cations (c = 10 mM) were prepared in de-ionized water (15 μM HEPES buffer, pH 7.2) for metal selectivity. For titration experiments, solutions of desired concentration of cations were prepared separately. For investigating the metal ion selectivity of the sensor **H₂PPC** and in titration experiments, quartz cells of 1 cm optical path length were filled with 2 mL solution of **H₂PPC** (20 μM) to which stock solutions of the metal ions were gradually added using a micropipette. UV– Vis and fluorescence spectra were recorded after 2 minutes of addition of metal salt. For fluorescence measurements, excitation was provided at 340 nm, and emission was acquired from 360 nm to 650 nm with 5 nm E_x bandwidth and 5 nm E_m bandwidth. All the measurements were taken at room temperature.

2.4 Limit of Detection (LOD) calculation

The limit of detection for Al^{3+} was calculated from the fluorescence titration applying the equation[24]

$$LOD = 3\sigma / \kappa$$

Where σ is the standard deviation of blank measurement, and k is the slope between the fluorescence emission intensity versus $[Al^{3+}]$. The slope was obtained from the plot of the fluorescence emission intensity at wavelength 506 nm vs. concentration of $[Al^{3+}]$.

2.5 Fluorescence quantum yield measurements

Fluorescence quantum yields (φ) were determined using the following equation:

$$\Phi_{sample} = \frac{OD_{standard} \; X \; A_{sample}}{OD_{sample} \; X \; A_{standard}} X \; \Phi_{standard}$$

Where A represents the area under the fluorescence spectral curve and OD symbolizes the optical density of the compound at the excitation wavelength 340 nm. Fluorescein in 0.1M NaOH (φ = 0.79) was taken as the reference compound to measure the fluorescence quantum yield.

2.6 Fluorescence lifetime measurements

Fluorescence lifetimes were measured by Time Correlated Single- Photon Counting (TCSPC) method using a HORIBA Jobin Yvon Fluorocube-01-NL fluorescence lifetime spectrometer. The sample was excited using a laser diode at 375 nm and the signals were collected at the magic angle of 54.7° to eliminate any considerable contribution from fluorescence anisotropy decay[25]. The typical time resolution of our

$$\tau_{av} = \frac{\sum \alpha_i \tau_i^2}{\sum \alpha_i \tau_i}$$

experimental setup is ~ 100 ps. The decays were deconvoluted using DAS-6 decay analysis software. Mean (average) fluorescence lifetimes were calculated using the following equation[26,27]:

in which α_i is the pre-exponential factor corresponding to the ith decay time constant, τ_i.

2.7 Computational details

Ground state electronic structure calculations in the gas phase of the ligand and complex have been carried out using the DFT[28] method associated with the conductor-like polarizable continuum model (CPCM).[29,30] Becke's hybrid function[31] with the Lee-Yang-Parr (LYP) correlation function[32] was used for the study. The absorbance spectral properties in MeOH medium for **H₂PPC** and **[Al(HPPC)₂]⁺(1)**, were calculated by time-dependent density functional theory (TDDFT)[33–35] associated with the conductor-like polarizable continuum model and we computed the lowest 40 singlet – singlet transition.

For H atoms we used 6-31(g) basis set; for C, N, O and Al atoms we employed 6-31+g as basis set for all the calculations. The calculated electron-density plots for frontier molecular orbitals were prepared by using Gauss View 5.1 software. All the calculations were performed with the Gaussian 09W software package.[36] Gauss Sum 2.1 program[37] was used to calculate the molecular orbital contributions from groups or atoms.

2.8 Cell Culture and Imaging Study

 (ia) Human lung cancer cell lines A549 were purchased from National Centre for Cell Science (NCCS, India) and was well maintained in heat-inactivated FBS (fetal bovine serum, 10%) containing Dulbecco's Modified Eagle's Medium (DMEM) added with 100 mg/mL concentration of antibiotics viz. penicillin, streptomycin, gentamycin and amphotericin B (fungizone). Cells were incubated in a chamber humidified with 5% CO₂ to achieve 70%–80% of confluence prior to the imaging experiment.

(ib) **In vitro live cell imaging of Al³⁺ in A549 cells**

In vitro imaging of Al³⁺ in live cells was executed according to previous methods.[38, 39] For in vitro imaging studies, the cells were seeded in 12-well tissue culture plates with a seeding density of 10^5 cells per well. After reaching 70%–80% confluence, the previous DMEM medium was replaced with serum free DMEM medium. Then **H₂PPC** (10 μM) was incubated for 2 hours to facilitate cellular uptake. After then the cells were washed three times with PBS to remove any free ligand present. After then, the cells were incubated further with Al³⁺ (50 μM) for 45 minutes in fresh serum-free DMEM. Images of live cells were then taken by using an EVOS® FL Cell Imaging System, Life Technologies, USA.

(iia) **Cell Culture of Vero Cells**

Vero Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 5–10% fetal bovine serum (FBS). A monolayer of cells (1.0×10^6 cells/ml) was grown onto 6-well plates in a humidified incubator at 5% CO_2 for 24 h at 37 °C. The cells were fixed in paraformaldehyde (4%) solution and blocked with 1% bovine serum albumin (BSA) in 0.1% PBS (phosphate-buffered saline)-Triton-X100 solution and were washed and then permeabilized with 0.1% Triton-X100 in PBS.

(iia) **In vitro fixed cell imaging of Al^{3+} in Vero cell**

The permeabilized Vero cell monolayer was treated with the ligand **H₂PPC** (50 µM) for 30 min and washed twice with PBS (pH 7.2) to eliminate cell debris.[39,40] The cells were subsequently incubated with an aqueous solution of Al^{3+} as $Al(NO_3)_3$ (100 µM) for 30 min, washed twice with PBS, and were observed under an epifluorescence microscope.

(iii) **Cytotoxicity assay:**

In vitro cytotoxicity was measured by using the colorimetric methyl thiazolyltetrazolium (MTT, Sigma-Aldrich, Germany) assay against A549 cells according to the previous report.[39,41] The Cells were seeded into 24-well tissue culture plate in presence of 500 µL Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37˚C temperature and 5 % CO_2 atmosphere for overnight and then incubated for 6-12 hours in presence of **H₂PPC** at different concentrations (10-100 µM). Then cells were washed with PBS buffer and 500 µL supplemented colorless DMEM medium was added. Subsequently, 50 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide MTT (5 mg/mL) was added to each well and incubated for 4 hours. Next, violet formazan was dissolved in 500 µL of sodium dodecyl sulfate solution in water/DMF mixture. In order to measure the viable number of cells, the absorbance of solution was measured at 570 nm using microplate reader. The numbers of viable cells were determined by analyzing the percent live cells against untreated controls. The individual values for three replicate determinations were calculated and their mean values are reported.

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