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Supporting Information

"Turn-on" fluorescence sensing of cytosine: Development of a chemosensor for quantification of cytosine in human cancer cells

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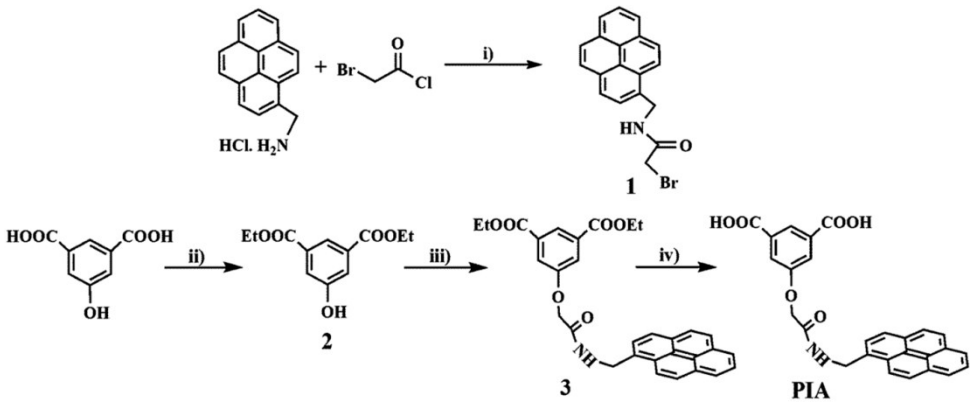
1. Experimental Section

Materials and Methods

1-Pyrenemethylamine hydrochloride, 5-Hydroxyisophthalic acid, Bromoacetyl chloride and cytosine were purchased from Sigma-Aldrich Pvt. Ltd. (India). Unless otherwise mentioned, materials were obtained from commercial suppliers and were used without further purification. Solvents were dried according to standard procedures. Elix Millipore water was used throughout all experiments. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker 400 MHz instrument. For NMR spectra, DMSO-d_6 and for NMR titration DMSO-d_6 and D_2O were used as solvent using TMS as an internal standard. Chemical shifts are expressed in δ ppm units and ^{1}H–^{1}H and ^{1}H–C coupling constants in Hz. The mass spectrum (HRMS) was carried out using a micromass Q-TOF Micro™ instrument by using Methanol as a solvent. Fluorescence spectra were recorded on a Perkin Elmer Model LS 55 spectrophotometer. UV spectra were recorded on a SHIMADZU UV-3101PC spectrophotometer. Elemental analysis of the compounds was carried out on Perkin-Elmer 2400 series CHNS/O Analyzer. The following abbreviations are used to describe spin multiplicities in ^{1}H NMR spectra: s = singlet; d = doublet; t = triplet; m = multiplet.

Synthetic Procedures

The fluorescent receptor **PIA** was synthesized by four consecutive steps starting from 1-Pyrenemethylamine hydrochloride followed by the preparation of compound **1**, **2** and **3** as shown in Scheme S1. Initially, **1** was synthesized according to a published procedure,[1] by the reaction of 1-Pyrenemethylamine hydrochloride and bromoacetyl chloride producing compound **1**. **PIA** has been synthesized from 5-Hydroxyisophthalic acid in three steps via formation of compound **2** and **3**. In the final step, compound **3** was hydrolysed to give the product **PIA** with 86% yield (Scheme S1).



Scheme S1. (i) Bromoacetyl chloride, K_2CO_3, water-ethyl acetate (1:1), rt, 2-3 h. (ii) Ethanol, conc. H_2SO_4, reflux, 12h. (iii) Compound **1**, acetonitrile, K_2CO_3, reflux, 24h. (iv) NaOH (10%) & EtOH (1:1), reflux, 24 h, H_3O^+.

2-Bromo-N-(pyren-1-ylmethyl)-acetamide (1): 1-pyrenemethylamine hydrochloride (0.804 g, 3 mmol) mixed with potassium carbonate (1.68 g, 12 mmol) is suspended into a mixture of ethyl

acetate (120 mL) and water (120 mL). Then, bromoacetyl chloride (7.07 g, 4.5 mmol) in ethyl acetate (10 mL) is added drop wise into the solution. After 2 h stirring at room temperature, the organic layer is isolated and dried by $MgSO_4$. The ethyl acetate solvent is removed by rotary evaporation to give the crude product that is purified by column chromatography (silica, 220–400 mesh, hexane/ EtOAc = 1:3 v/v). The product is isolated as a white powder **1** (0.76 g, 72%). ^{1}H-NMR (DMSO-d_6, 400 MHz): δ (ppm) 4.41 (s, 2H), 5.05 (d, J = 8 Hz, 2H), 8.07–8.33 (m, 9H), 8.92 (t, J = 8 Hz, 1H); ^{13}C-NMR (DMSO-d_6, 400 MHz): δ (ppm) 30.1, 123.7, 124.3, 124.5, 125.2,125.7, 125.8, 126.8, 127.3, 127.6, 127.8, 128.3, 128.6, 130.7, 131.2, 132.7, 167.0. ESI/MS: m/z calcd for $C_{19}H_{14}BrNO$: 352.02, found $[M+H]^+$: 353.04. Anal. Calcd for $C_{19}H_{14}BrNO$: C, 64.79; H, 4.01; N, 3.89. Found: C, 64.88; H, 4.07; N, 3.81.

Di-ester of 5-Hydroxyisophthalic acid: To a solution of anhydrous ethanol (30ml) 5-Hydroxyisophthalic acid (1.82g, 10 mmol) was added. Catalytic amount of conc. sulfuric acid was then added to the reaction mixture. The mixture was then stirred for 24 hrs. at 80^{0}C. The solution was extracted with chloroform (3×20 mL) and water (3×50 mL). Organic layer was separated and dried over by anhydrous $MgSO_4$. The solvent was removed by rotary evaporation to give the di-ester product (yield 98%).

PIA: To a solution of anhydrous K_2CO_3 (0.85g, 6 mmol) in dry acetonitrile was added di-ester of 5-Hydroxyisophthalic acid (0.36 g, 1.50 mmol). The mixture was stirred for 0.5 h. Then compound **1** (1.06 g, 2 mmol) was added to the solution and stirred for 48 h. Then, the reaction mixture was poured into water. The solution was extracted with chloroform (3×50 ml). The organic layer was separated and dried over anhydrous $MgSO_4$. After removing the solvents, the residue was chromatographed on silica gel with chloroform/ Ethyl acetate = 4:1 v/v as eluent to give 0.31g (86%) of compound **3**. The diester was dissolved in a mixture of 10% aqueous NaOH solution and EtOH (1:1) under reflux for 24 h. The reaction mixture was evaporated and it was acidified with conc. HCl into ice. The brown ppt was collected and dried at room temp (0.3 g, 98%). 1H NMR (DMSO-d6, 400 MHz): δ (ppm) 4.77-4.83 (m, 4H), 8.07-8.14 (m, 2H), 8.20-8.25 (m, 4H), 8.32-8.38 (m, 6H), 8.46-8.48 (t, 1H), 8.74 (s, 2H). ^{13}C-NMR (DMSO-d6, 400 MHz): δ (ppm) 12.81, 63.75, 124.05, 124.69, 125.80, 126.56, 126.71, 127.54, 128.30, 128.59, 128.91, 128.98, 129.13, 129.68, 131.25, 131.72, 132.06, 158.54, 167.24. HRMS (TOF MS): (m/z, %): 454.1130 [(M + H^+), 100 %]; Calculated for $C_{27}H_{19}O_6N$: 453.10514. Anal. Calcd for $C_{27}H_{19}O_6N$: C, 71.6; H, 4.13; N, 3.09; O, 21.18; Found: C, 71.7; H, 4.03; N, 3.08; O, 21.19.

2. Table S1. Performance comparison of existing methods and present method for detection of cytosine

Method of detection	Detection limit	Response Time	Exogeneous detection	Endogeneous detection	Quantification in simple or complex matrix	Water soluble and cost effective	References
Fluorescence	**32 nM**	**In few Minutes**	**Yes**	**Yes**	**Yes both in simple and complex**	**Yes**	**Present manuscript**
Electrochemical	2000 nM	Several hours	Yes	No	Only simple	No	J Solid State Electrochem. 20 (2016) 2223.
HPLC–DAD-MS	0.1 - 0.3 μg/ml	Few hours	Yes	No	Complex with tissue extract	No	Journal of Pharmaceutical and Biomedical Analysis 44 (2007) 807–811.
HPLC–ESI–MS/MS	3.45 ng/ml	Few hours	Yes	No	Simple and complex with tissue extract	No	Analytica Chimica Acta 567 (2006) 218–228
Nanopore based technology (Biosensor)	ND	Few hours	Yes	No	Simple	No	Nature Nanotechnology 6 (2011) 615-624. And references therein.

3. NMR Spectral studies

^{1}H NMR of compound 1 in DMSO-d_6:

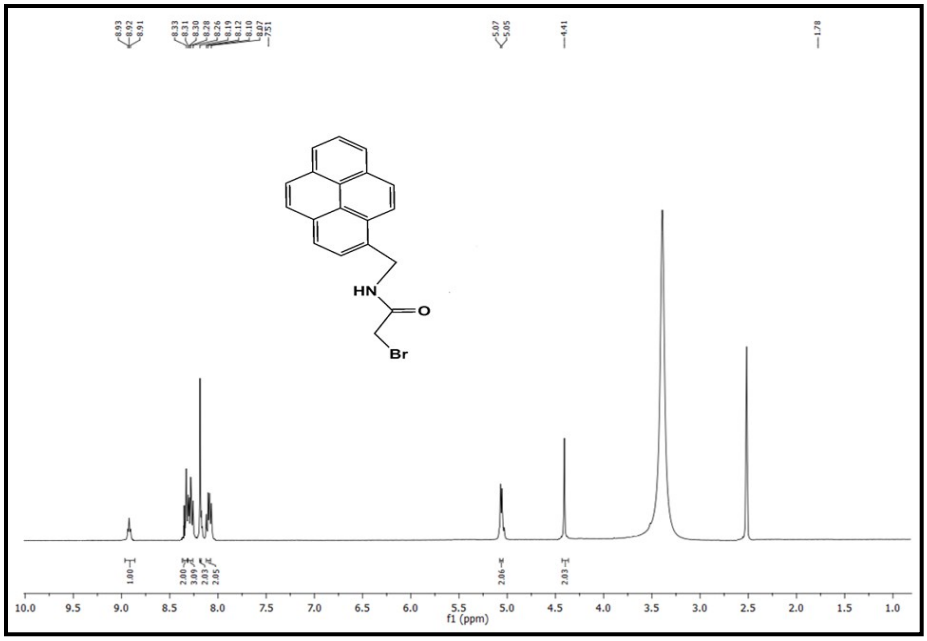


Fig. S1 ^{1}H NMR of compound **1** in DMSO-d_6 (400 MHz).

^{13}C NMR of compound 1 in DMSO-d_6:

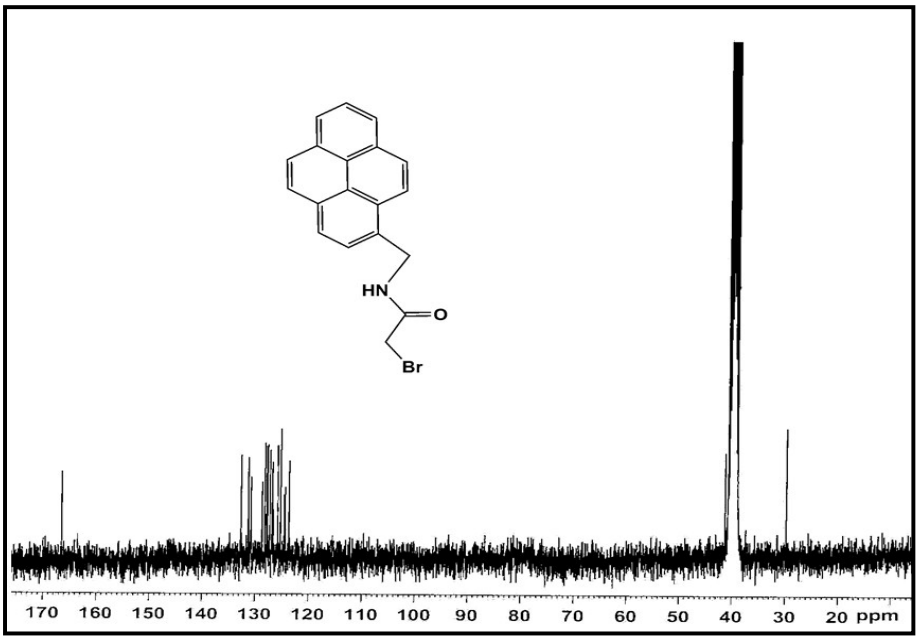


Fig. S2 ^{13}C NMR of compound **1** in DMSO-d_6 (400 MHz).

^{1}H NMR of PIA in DMSO-d_6:

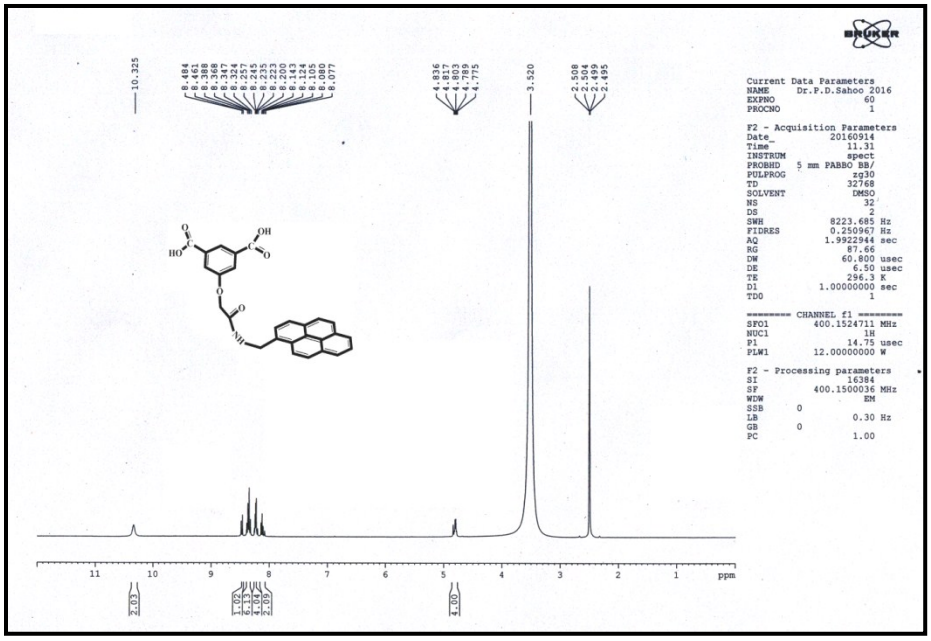


Fig. S3 ^{1}H NMR of **PIA** in DMSO-d_6 (400 MHz).

^{13}C NMR of PIA in DMSO-d_6:

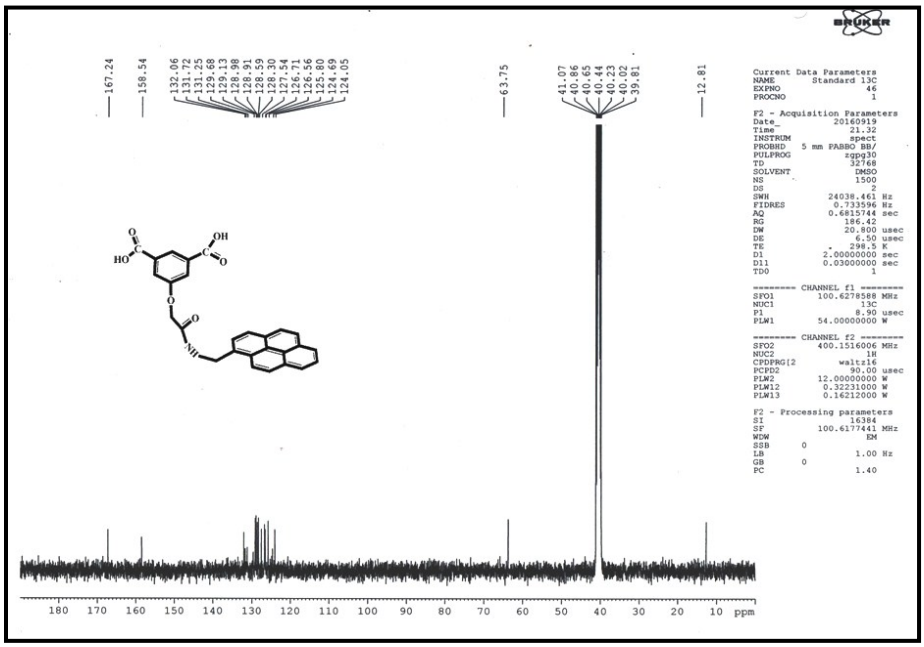


Fig. S4 ^{13}C NMR of **PIA** in DMSO-d_6 (400 MHz).

4. Mass spectrum of PIA :

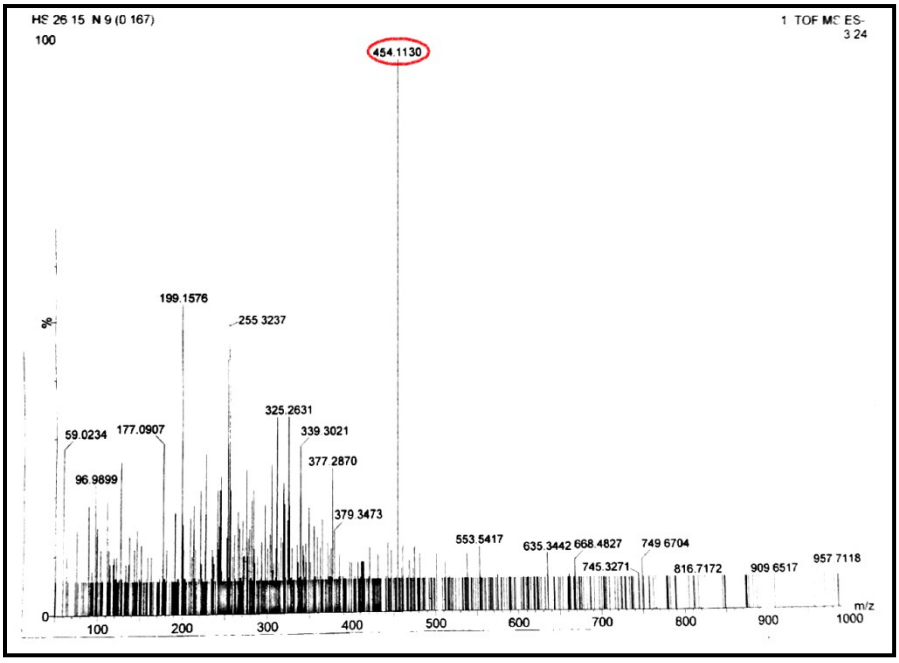


Fig. S5 HRMS of **PIA**.

5. UV-Vis and fluorescence titration studies

UV-vis spectral studies:

A stock solution of **PIA** (1×10^{-6} M) was prepared in water-DMSO (20:1, v/v). Cytosine solution of concentration 1×10^{-5} M was prepared in Millipore water. All experiments were carried out in aqueous medium at neutral pH. During **PIA** and cytosine titration, each time a 1×10^{-6} M solution of **PIA** was filled in a quartz optical cell of 1 cm optical path length and cytosine stock solution was added into the quartz optical cell gradually by using a micropipette. Spectral data were recorded at 1 min after the addition of cytosine.

Fluorescence spectral studies:

A stock solution of **PIA** (1×10^{-6} M) was prepared in water-DMSO (20:1, v/v). Cytosine solution of concentration 1×10^{-5} M was prepared in Millipore water. All experiments were carried out in aqueous medium at neutral pH. During **PIA** and cytosine titration, each time a 1×10^{-6} M solution of **PIA** was filled in a quartz optical cell of 1 cm optical path length and cytosine stock solution was added into the quartz optical cell gradually by using a micropipette. Spectral data were recorded at 1 min after the addition of cytosine. For all fluorescence measurements, excitations were provided at 345 nm, and emissions were collected from 365 to 460 nm.

6. Measurement of fluorescence quantum yields

The fluorescence quantum yield (QY) of **PIA** was determined relative to a reference compound of known QY. 2-Aminopyridine (solvent 0.1 M H_2SO_4) was chosen as reference compound because it has emission profile between 320-480 nm similar to the receptor **PIA**. The quantum yield of **PIA** increased almost 2.5 fold upon addition of 1 equiv. of cytosine.

7. Evaluation of the association constants for the formation of (PIA-cytosine) complex:

By Fluorescence Method:

Binding constant of the chemosensor **PIA** was calculated through emission method by using the following equation:

$$1/ (I - I_0) = 1/K(I_{max} - I_0) [G] + 1/(I_{max} - I_0) \quad \text{……..(ii)}$$

Where I_0, I_{max}, and I represent the emission intensity of free **PIA**, the maximum emission intensity observed in the presence of added cytosine at 377 nm (λ_{ex}= 345 nm), [G] is the concentration of the guest cytosine and the emission intensity at a certain concentration of the cytosine, respectively. [H] is the concentration of the host **PIA**.

Binding constant calculation graph (Fluorescence method):

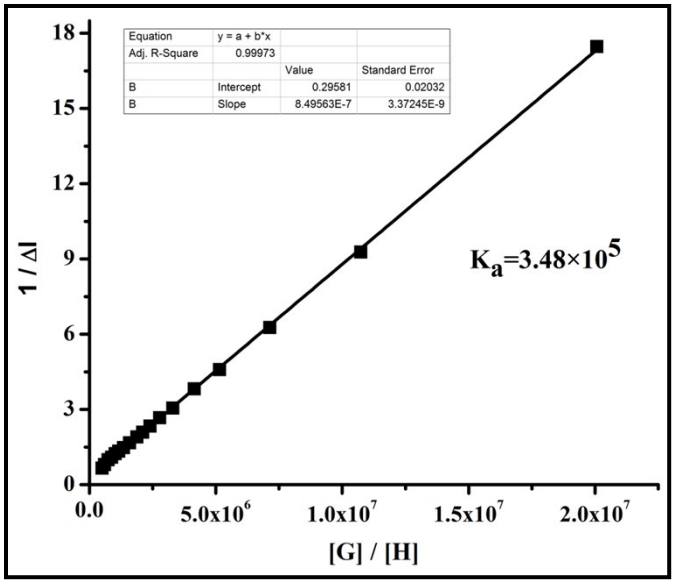


Fig. S6 Linear regression analysis (1/[G] vs 1/ΔI) for the calculation of association constant values by Fluorescence titration method.

The association const. (K_a) of **PIA** for sensing cytosine was determined from the equation: K_a = intercept/slope. From the linear fit graph we get intercept= 0.29581, slope = 8.49563 × 10^{-7}. Thus we get, K_a = (0.29581) / (8.49563× 10^{-7}) = 3.48 × 10^5 M^{-1}.

8. Job's plot for determining the stoichiometry of binding by fluorescence method:

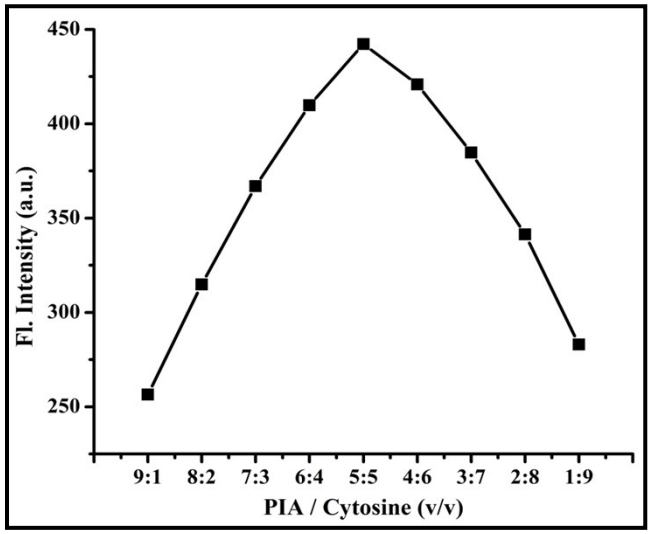


Fig. S7 Job's plot of **PIA** (c = 1 × 10^{-6} M) with cytosine (c = 1 × 10^{-6} M) in water-DMSO (20:1, v/v) at neutral pH by fluorescence method, which indicate 1:1 stoichiometry for **PIA** with cytosine.

9. Calculation of limit of detection (LOD) of PIA with cytosine:

The detection limit of the receptor **PIA** for cytosine was calculated on the basis of fluorescence titration. To determine the standard deviation for the fluorescence intensity, the emission intensity of four individual receptors without cytosine was measured by 10 times and the standard deviation of blank measurements was calculated.

The limit of detection (LOD) of **PIA** for sensing cytosine was determined from the following equation[2-3]:

$$LOD = K \times SD/S$$

Where K = 2 or 3 (we take 3 in this case); SD is the standard deviation of the blank receptor solution; S is the slope of the calibration curve.

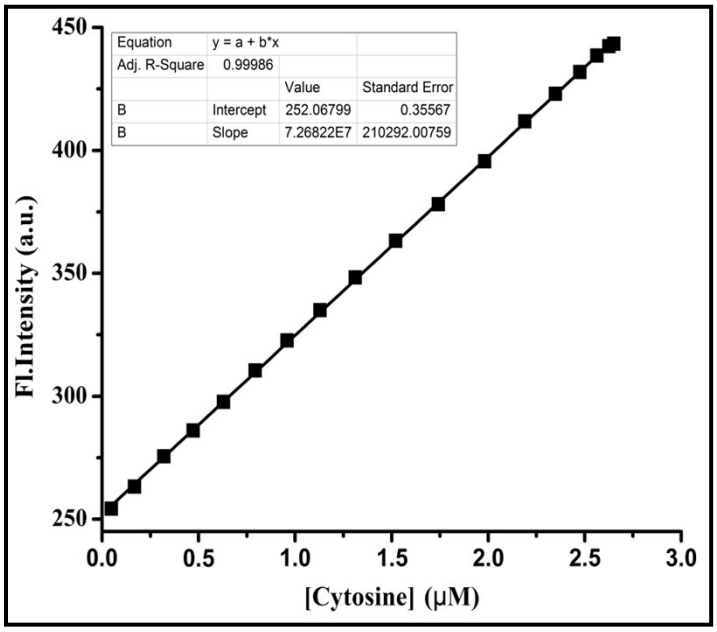


Fig. S8 Linear fit curve of **PIA** at 377 nm with respect to cytosine concentration.

For **PIA** with cytosine:

From the linear fit graph we get slope = 7.26822×10^7, and SD value is 0.79107.

Thus using the above formula we get the Limit of Detection = 0.32×10^{-7} M, i.e 32 nM. Therefore **PIA** can detect cytosine up to this very lower concentration by fluorescence techniques.

10. Competitive fluorescence titration studies of PIA with all pyrimidine/purine bases:

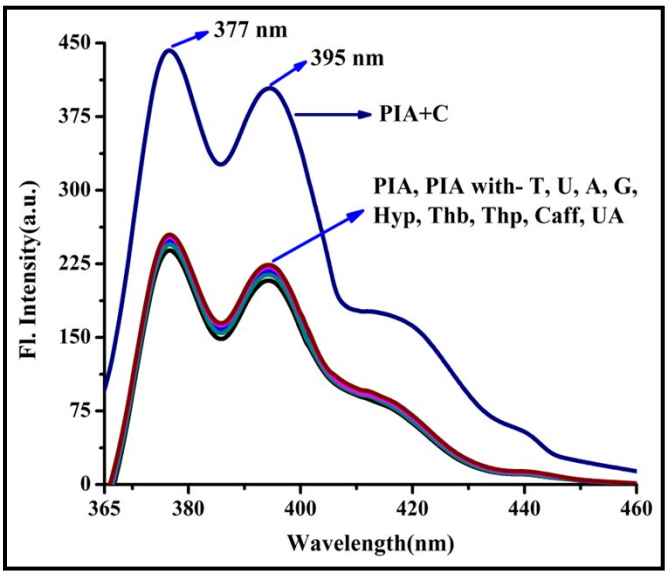


Fig. S9 Fluorescence emission spectra (λ_{ex}= 345 nm) of **PIA** (1 μM) upon addition of 1.2 equiv. of various pyrimidine/purine derivatives (e.g. cytosine, thymine, uracil, adenine, guanine, hypoxanthine, theobromine, theophylline, caffeine and uric acid) in water-DMSO (20:1, v/v) at neutral pH.

11. DFT Study:

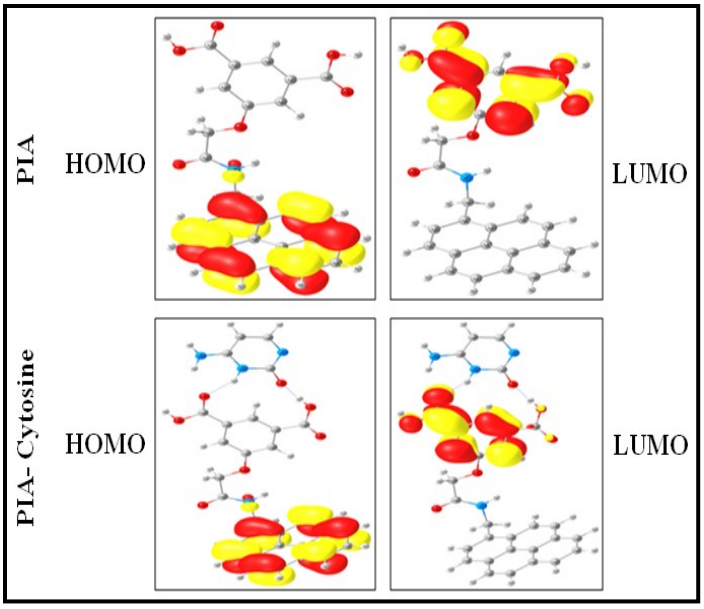


Fig. S10 Molecular orbitals and electronic contributions of the relevant excitations of **PIA** and **PIA-**cytosine complex.

Table S2. Selected electronic excitation energies (eV), oscillator strengths (f), main configurations of the low-lying excited states of all the molecules and complexes. The data were calculated by TDDFT//B3LYP/Def2SVP based on the optimized ground state geometries

Molecules	Electronic Transition	Excitation Energy[a]	f[b]	Composition[c]
Cytosine	$S_0 \rightarrow S_2$	4.808 eV 257.9 nm	0.1710	H → L (90%)
	$S_0 \rightarrow S_7$	6.478 eV 191.4 nm	0.1216	H-3 → L (26%) H → L +2 (39%)
PIA	$S_0 \rightarrow S_3$	3.805 eV 325.8 nm	0.3588	H → L +1 (81%)
	$S_0 \rightarrow S_{14}$	4.781 eV 259.9 nm	0.2998	H-1 → L +1 (33%) H → L +3 (31%)
PIA-Cytosine	$S_0 \rightarrow S_5$	3.806 eV 325.7 nm	0.3453	H → L +1 (79%)
	$S_0 \rightarrow S_{22}$	3.805 eV 260.0 nm	0.2059	H-2 → L +1 (24%) H → L +3 (21%)

[a] Only selected excited states were considered. The numbers in parentheses are the excitation energy in wavelength. [b] Oscillator strength. [c] H stands for HOMO and L stands for LUMO.

Table S3. Energies of the highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO)

Species	**E_{HOMO} (a.u)**	**E_{LUMO} (a.u)**	**ΔE(a.u)**	**ΔE(eV)**	**ΔE(kcal/mol)**
Cytosine	-0.219685	-0.033747	0.185938	5.059670481	116.6778614
PIA	-0.204055	-0.072666	0.131389	3.575304912	82.4478457
PIA-Cytosine	-0.201283	-0.083269	0.118014	3.211349762	74.05490613

Computational details: Geometries have been optimized at the B3LYP/Def2SVP level of theory. The dispersion corrections have also been incorporated using Grimme's D3 with Becke−Johnson damping (GD3BJ) algorithm have been included.[4-5] The geometries are verified as proper minima by frequency calculations. Time-dependent density functional theory calculation has also been performed at the same level of theory. All the calculations have been carried out using ORCA software suite.[6]

12. ^{1}H NMR titration spectrum of PIA with cytosine:

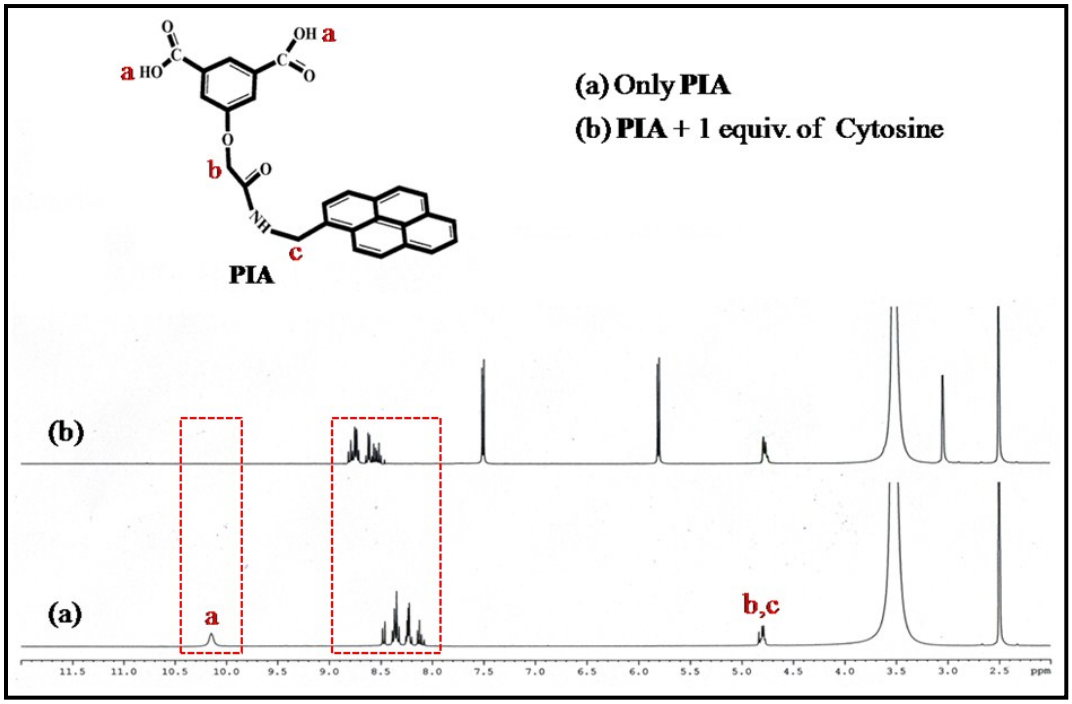


Fig. S11 ^{1}H NMR titration [400MHz] spectra of **PIA** in DMSO-d_6 at 25^{0}C and the corresponding changes after the addition of 1 equiv. of cytosine in DMSO-d_6 from (a) only **PIA**, (b) **PIA** + 1 equiv. of cytosine.

13. Live Cell Imaging

Cell line and cell culture

Cell Culture: A549 cell (Human cell A549, ATCC No CCL-185) lines were prepared from continuous culture in Dulbecco's Modified Eagle's Medium (DMEM, Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum (Invitrogen), penicillin (100 µg/mL), and streptomycin (100 µg/mL). Cells were initially propagated in 75 cm^2 polystyrene, filter-capped tissue culture flask in an atmosphere of 5% CO_2 and 95% air at 37°C in CO_2 incubator. When the cells reached the logarithmic phase, the cell density was adjusted to 1.0 ×10^5 per/well in culture media. The cells were then used to inoculate in a glass bottom dish, with 1.0 mL (1.0 ×10^4 cells) of cell suspension in each dish. After cell adhesion, culture medium was removed. The cell layer was rinsed twice with phosphate buffered saline (PBS) (pH 7.0), and then treated according to the experimental need.

Cell imaging study

For confocal imaging studies, 1×10^4 A549 cells in 1000 µL of medium, were seeded on sterile 35 mm glass bottom culture dish (ibidi GmbH, Germany), and incubated at 37°C in a CO_2 incubator for 10 hours. Then cells were washed with 500 µL DMEM followed by incubation with **PIA** (1 µM) dissolved in 1000 µL DMEM at 37°C for 1 h in a CO_2 incubator and cells were washed thrice with phosphate buffered saline (PBS) (pH 7.0) to remove excess **PIA** observed under an Olympus IX81 microscope equipped with a FV1000 confocal system using 1003 oil immersion Plan Apo (N.A. 1.45) objectives. Images obtained through section scanning were analyzed by DIDS with excitation at 341 nm monochromatic laser beams, and emission spectra were integrated over the range 414 nm (single channel). The cells were again incubated with cytosine (10 µM) for 20 min and excess cytosine was thrice with PBS (pH 7.0) followed by observations under microscope. For all images, the confocal microscope settings, such as transmission density, and scan speed, were held constant to compare the relative intensity of intracellular fluorescence.

14. Cytotoxicity Assay

In vitro studies established the ability of the chemosensor **PIA** to detect cytosine in biological system with excellent selectivity. Human cell A549 (ATCC No CCL-185) were used as models. However, to materialize this objective, it is a prerequisite to assess the cytotoxic effect of **PIA** and **PIA**-cytosine complex on live cells. The well-established MTT assay[7] was adopted to study cytotoxicity of above mentioned complexes at varying concentrations detailed in method section. A cytotoxicity measurement for each experiment shows that the chemosensor **PIA** does not have any toxicity on the tested cells and **PIA**-cytosine complex does not exert any significant adverse effect on cell viability at tested concentrations.

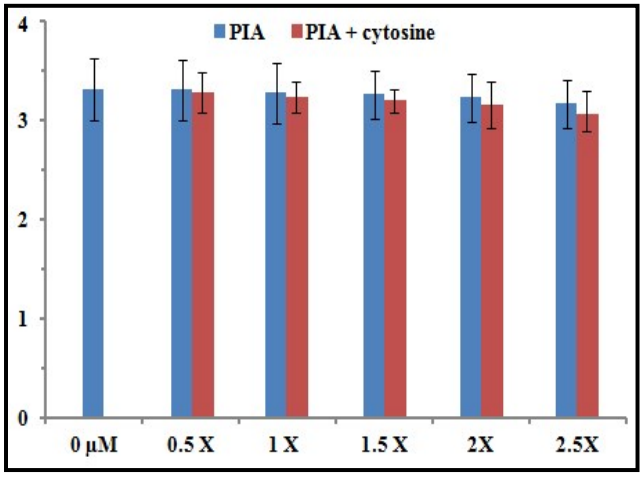


Fig. S12 MTT assay to determine the cytotoxic effect of **PIA** and **PIA**-cytosine complex on A549 cells (Human cell A549, ATCC No CCL-185).

15. Quantification of cytosine and validation of the screening procedure

To quantify cellular level of cytosine 10^7 A549 human cancer cells were harvested by centrifugation at 3000 rpm for 5 minutes followed by washing of the cell pellet with PBS buffer. Cells were again harvested following similar centrifugation. Cell pellet were suspended with 100 µL cold deionized water in order to lyse by the osmotic shock. Lysates were further centrifuged and the supernatant has been collected. The supernatant has been added with 1µM **PIA** and the fluorescence signal was measured. The value of fluorescence intensity has been plotted to the standard curve in order to know the concentration of cytosine in tested sample (Fig. 5). All estimations have been done in triplicate.

The estimation of cytosine was validated using A549, HeLa and Hep-2 cancer cells. 10^4 of each cell suspension were centrifuged to collect the cells. The cells were resuspended with 10 mM PBS buffer (pH 7.0) followed by centrifugation. The cell pellets were lysed by osmotic shock with 100 µL ice cold deionised water. Supernatant were added with 1 µL **PIA** and fluorescence signal were recorded. The fluorescence signal has been recorded for five independent sample of each cancer cell type and all experiments were done in triplicate. The signal to noise ratio were obtained and the screening procedure were validated by calculating Z' score (Table S4).

16. Table S4. Optimization and validation of the screening procedure for cytosine level in various biological samples using **PIA** chemosensor

Different Cancer cells	Samples	Fluorescence Intensity			Mean	Standard Deviation	Signal*/Noise**	Z' score
		Set 1	Set 2	Set 3				
A549 Cell	**Control ($c = 1 \times 10^{-6}$ M)**	226	230	227	227.66	2.08	----	----
	S 1	442	444	447	444.33	2.51	1.95	0.93
	S 2	444	441	442	442.33	1.52	1.94	0.94
	S 3	446	452	447	448.34	3.21	1.96	0.92
	S 4	442	444	441	442.33	1.52	1.94	0.94
	S 5	444	442	447	444.33	2.51	1.95	0.93
HeLa Cell	**Control ($c = 1 \times 10^{-7}$ M)**	210	215	211	212	2.64	----	----
	S 1	400	398	401	399.66	1.52	1.88	0.93
	S 2	408	403	405	405.33	2.51	1.91	0.92
	S 3	414	417	418	416.33	2.08	1.96	0.93
	S 4	430	428	431	429.66	1.52	2.02	0.94
	S 5	428	430	434	430.66	3.05	2.03	0.92
Hep-2 Cell	**Control ($c = 1 \times 10^{-4}$ M)**	293	296	292	293.66	2.08	----	----
	S 1	553	555	556	554.66	1.52	1.88	0.96
	S 2	565	568	573	568.67	4.041	1.93	0.94
	S 3	566	569	571	568.66	2.51	1.93	0.95
	S 4	586	589	588	587.66	1.52	2.00	0.96
	S 5	580	586	584	583.3	3.05	1.98	0.95

*Fluorescence intensity for **PIA**-cytosine interaction.

Fluorescence intensity for **PIA.

17. References

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