CHAPTER 3

Selective recognition of uric acid in live cells using a pyrene appended thymine derivative
3.1. Introduction

Uric acid (UA) is the primary end product of purine metabolism and excreted in urine. Exogenously, non-vegetarian food and caffeine present in tea or coffee are the major sources of UA (Figure 3.1). It is a weak organic acid exists mainly as a monosodium salt under physiological conditions. UA is derived from purines arising from the catabolism of dietary and endogenous nucleic acids. This increased catabolism dysfunction of one of the shunt pathways sometimes leads to increased UA production.\(^1\)

![Figure 3.1. Structure of uric acid](image)

Enzyme xanthine oxidase catalyzes the formation of UA from xanthine and hypoxanthine, which in turn are produced from other purines.\(^2\) The level of UA in biological fluids is a balance between its synthesis in the liver and excretion in urine.\(^3\) Two thirds of the urate formed each day is excreted by the kidneys which reclaim most of the UA filtered at the glomeruli as it has excellent antioxidant capacity due to the presence of double bonds, and it can be responsible for 2/3 of the total plasma antioxidant capacity.\(^4\) Thus it can protect cells from damage by reactive oxygen species (ROS) and one-third is eliminated via the gastrointestinal tract. Excessive production of UA may lead to precipitation in the kidney and lower extremities.\(^5\) Although UA is excreted by the kidneys, but sometimes due to urine accumulation, there may be crystallization of UA in the form of insoluble calcium urate which leads to UA stone formation and if the excretion continues to be excessive, the kidneys may even be damaged markedly. Normally, UA is present in the blood in concentration range 0.15–0.45 mmol L\(^{-1}\) and excreted in urine in 1.19–2.98 mmol/day.\(^6\) UA levels are influenced by age and sex. Prior to puberty, the average serum UA is 3.4–7.2 mg dl\(^{-1}\) for males and 2.4–6.1 mg dl\(^{-1}\) for females. Following puberty, values rise to adult levels with women typically 1 mg dl\(^{-1}\) less than men. This lower level in women apparently reflects estrogen related enhancement of renal urate clearance. Many additional factors, including exercise, diet, state of hydration results in transient fluctuations of UA levels.
Clinical significance: The concentration of UA in body fluids (e.g. serum and urine) is extremely important diagnostic and prognostic factor of many multifactorial disorders. One of the major sites where the anti-oxidant effects of UA have been proposed is in the central nervous system, particularly in conditions such as multiple sclerosis, Parkinson’s disease, and acute stroke. UA concentrations in blood plasma above and below the normal range (200–430 µmol/L for males and 140–360 µmol/L for females) are known as hyperuricemia and hypouricemia, respectively.

Figure 3.2. Diseases related to hyperuricemia: (a) gout in hands, (b) formation of UA crystals in gout, (c) formation of kidney stones, (d) extreme nail biting caused by Lesch–Nyhan syndrome.
The physiochemical definition of hyperuricemia may be considered as 7.0 mg dl\(^{-1}\), measured by the specific uricase method. This represents the solubility limit of urate in plasma at 37°C. Levels beyond 7.0 results in supersaturated solutions that is prone to crystal formation. High intake of dietary purine, high–fructose corn syrup, and table sugar can increase levels of UA.\(^8\) Fasting or rapid weight loss can temporarily elevate UA levels. UA levels in saliva possibly associated with blood UA levels.\(^9\) The presence of elevated UA levels is a sign of gout (a condition where UA precipitates in the joints leading to pain and inflammation), hyperuricemia, or Lesch–Nyhan syndrome (Figure 3.2).\(^10\) Similarly, elevated UA levels are related to other conditions including hypertension, obesity, diabetes, high cholesterol, acute renal disease, liver disease (such as cirrhosis), hyperlipidemia, hypothyroidism, multiple sclerosis, toxemia of pregnancy, leukemia, chronic kidney disease, heart diseases and stroke.\(^11\) Many epidemiological studies have suggested that serum UA is also a risk factor for cardiovascular disease.\(^12\) In fact, patients with gout have a high risk of death, mainly due to cardiovascular causes.\(^13\) UA is released in high altitude hypoxic conditions.\(^14\) An excess level of UA is related with type–II diabetes.\(^15\) Hyperuricemia is associated with increased risk of colorectal, breast, prostate cancer risk, recurrence, and mortality.\(^16\) Inflammatory stress induced by elevated intracellular UA may contributes to tumorigenesis by promoting transformation, while elevated extracellular UA may further stimulate tumor cell proliferation, migration, and survival contributing to the development of highly aggressive cancer.\(^17\)

### 3.2. Previous work

Different research groups have been actively engaged in the study of recognition of UA for its exceptional importance in clinical as well as diagnostic research. Kelly et al. first reported a system, which not only restricted UA as a guest to a particular position with respect to the host but also demonstrated the substantial improvement of binding affinity. They synthesized and described the binding pattern of UA with synthetic rigid receptors (Figure 3.3). Receptor 1 was designed to recognize UA; CPK models indicated that an excellent fit was achieved.\(^18\) It was quite hard to determine whether binding of receptor 1 with UA occurs, because UA is very much insoluble in organic solvents. They prepared a
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derivative of UA i.e. \( N \)-dodecyl UA, which was sufficiently soluble to allow binding studies in a polar solvent like DMSO.

![Chemical structure](image)

**Figure 3.3.** Recognition pattern of UA by receptor 1.\(^{18}\)

There are many standard analytical techniques in the literatures for UA determination. The levels of UA in different biological matrices such as serum and urine have been determined by various standard analytical methods such as potentiometric enzyme electrode,\(^{19}\) reversed–phase liquid chromatography,\(^{20}\) and flow injection analysis system with tubular amperometric detector.\(^{21}\) Hamzah et al. has reported a simple spectrophotometric method for detection of UA in urine, based on enzymatic reaction of uricase, hydrogen peroxide and 4-aminodiphenylamine diazonium sulfate (Variamine Blue RT salt) (Figure 3.4).\(^{22}\)

![Chemical structure](image)

**Figure 3.4.** (a) Oxidation of UA by uricase enzyme in the presence of Variamine Blue RT. (b) absorption spectra of the enzymatic reaction uricase–UA in absence and presence of Variamine Blue RT salt; green line: uricase + UA; blue line: uricase + Variamine Blue RT salt (blank); red line: uricase + UA + Variamine Blue RT salt.\(^{22}\)
Martinez-Pérez et al. has developed a reagent-less and ready-to-use fluorescent biosensor for the detection of UA in biological fluids. Bera et al. developed a non-enzymatic analytical method for the selective detection and quantification of serum UA using 2-thiouracil functionalized Au nanoparticles. It showed an instant visible color change due to H–bonding interaction between amide moieties of UA with four and six 2-thiouracil units. The aggregation of the functionalized Au nanoparticles effectively decreases the inter particle distance and induces visible color change (Figure 3.5). Hydrophobic and π–π interactions also play an essential role in this context.

![Figure 3.5](image)

**Figure 3.5.** H–bonding interaction of 2-thiouracil adapted Au nanoparticles with UA; the potential interaction of four and six 2-thiouracil units are shown in (a) and (b).

However, these detection methods have major limitations such as they are still under growth, time–consuming, expensive, cannot be executed easily outside the laboratory and also require very highly practiced technicians. Also estimating the level of UA in a clinical laboratory is complicated because of the enzyme and reagents concerned. Among these reported methods, fluorescent probes possess innate advantages over probes of other types because of their high sensitivity, selectivity, specificity, convenience, fast on-site evaluation, low cost, simplicity of implementation and fast response times.
Recently, Dey et al. reported naphthyridine–based receptors (R1, R2, R3 and R4) for detection of UA. The interactive properties of the receptor–UA complex were verified by \(^1\)H NMR, UV–vis and fluorescence studies. UA contains hydrogen bond donor (D)–acceptor (A) patterns such as DADA and ADDA in a cyclic arrangement. They used ADAD and DAAD type receptors, which were designed for positive complexation. The stability of host–guest assemblies were established by minimization energy calculations and molecular modeling studies (Figure 3.6).\(^{27}\)

![Figure 3.6. Structure of naphthyridine–based receptors and corresponding energy minimized structures of receptor–UA complexes.\(^{27}\)](image)

### 3.3. Objectives

Complexation of neutral biomolecules have been extensively studied in order to understand their behavior in complex biological systems.\(^{28}\) Hydrogen bonding via complementary base pairing plays fundamental roles in bio molecular recognition, such as replication of nucleic acids, maintenance of tertiary structure of proteins and enzymatic substrate recognition.\(^{29}\)

Design of artificial chemosensors to specifically target biologically important small molecules in aqueous media is a challenging task. Purines and pyrimidines both are heterocyclic, polar and water–soluble. Therefore it is more challenging to develop a chemosensor for selective recognition of UA over other purine derivatives, because of the less solubility of UA in water and other polar organic solvents. Several studies using synthetic receptors in polar organic solvents have shown that hydrogen bonding alone or combined with aromatic stacking are the most significant binding forces.\(^{30}\) The hydrogen
bonding involved in the base–pairing is a highly directional secondary valence force compared with other non-covalent bonding such as electrostatic, van der Waals and hydrophobic interactions. This directionality in intermolecular interactions is crucial for specific molecular recognition. In fact, hydrogen bonding has been used effectively in artificial recognition systems.

The “turn–on” chemosensors have a number of advantages such as (a) reduces the chance of a false positive, observed in some “turn–off” probes, (b) allows for the use of multiple probes, selective for different analytes, and (c) applicable in the analysis of both, aqueous environmental and biological samples. In this context our major objectives are:

- To design and synthesize of a “turn–on” fluorescent chemosensor for selective recognition of UA.
- To characterize the synthesized chemosensor by various analytical and spectral techniques.
- To study the recognition pattern of the chemosensor–UA complexation by several spectroscopic experiments.
- To investigate the stability of the chemosensor in the formation of host–guest complex by performing energy calculations.
- To understand the permeability and toxicity of the chemosensor inside live cells.
- To explore the newly synthesized chemosensor for its usage in live-cell imaging to visualize the presence of UA.

### 3.4. Present work

Here, in this work we prepared a new and simple chemosensor based on pyrene appended thymine derivative (PTA), which showed efficient fluorometric selectivity for UA in water. This receptor contains the hydrogen–bond donor–acceptor groups (thymine acetamide) for favorable complexation and the fluorophore pyrene makes π-stacking with the purine moiety of guest molecule UA. The receptor exhibits remarkable fluorescence intensity change in presence of the UA while the other purine bases which are structurally similar to UA remain unchanged. To the best of our knowledge, this is the first report, where we explored the possibility of using water soluble pyrene based fluorescent probe for the in vivo recognition of the UA in living cells using confocal imaging experiments.
3.4.1. Synthesis of the chemosensor

The synthesis of fluorescent probe PTA was carried out by two consecutive steps (Scheme 3.1), started with the preparation of 2-bromo-N-(pyren-1-ylmethyl)acetamide (1), which was obtained using standard reaction between 1-pyrenemethylamine hydrochloride and bromo acetyl chloride. Formation of two amide groups in PTA afforded the key interaction with UA through hydrogen bond donor–acceptor pattern in a cyclic array. All the precursor and probe molecules were characterized by various analytical and spectral techniques.

![Scheme 3.1. Preparation of PTA (a) EtOAc, K$_2$CO$_3$, 2 h, RT (b) K$_2$CO$_3$/DMF, 48 h.](image)

3.4.2. Results and discussion

In order to ascertain the interaction of PTA with UA, spectrophotometric and spectrofluorimetric titrations were carried out by adding varying concentrations of UA to a fixed concentration (10 μM) of PTA in aqueous solution (pH 7.4, 10 mM HEPES buffer).

3.4.2.1. UV–vis and fluorescence spectroscopic studies

The UV–vis absorption spectra of the chemosensor PTA in water (10 mM HEPES buffer, pH 7.4) subjected by an absorption band at 292 nm, was gradually enhanced, while the little decreased intensity of absorption showed a blue shift from 330 nm to 346 nm correspondingly upon gradual addition of UA (Figure 3.7.a), indicating the interaction of amide moiety of UA with two other amide groups of PTA. Two clear isosbestic points observed at 283 nm and 315 nm indicate the formation of host–guest complex structure with 1:2 stoichiometry. The interaction of PTA with UA was also studied by fluorescence spectroscopy (Figure 3.7.b). UA (100 μM) was added to a buffered aqueous solution (pH 7.4) of PTA (10 μM), a pronounced increase in fluorescence intensity were observed at 377
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nm and 395 nm ($\lambda_{ex} = 315$ nm) while performing the fluorescence titration experiment with UA concentrations up to 2 equivalent.

**Figure 3.7.** (a) UV–vis titration spectra of PTA (10 μM) with UA (100 μM) in water at neutral pH, (b) fluorescence emission spectra of the chemosensor PTA (10 μM) with UA (100 μM) in water at neutral pH ($\lambda_{ex} = 315$ nm).

### 3.4.2.2. Selectivity studies

In order to understand the binding potency and sensing selectivity of the chemosensor PTA, parallel investigations were carried out with a variety of biologically relevant purine bases such as adenine, guanine, caffeine, theophylline and hypoxanthine, which are structurally similar to UA. Figure 3.8 displays the changes in fluorescence of PTA upon addition of various purine bases under a UV lamp. As in the presence of UA, a marked change in fluorescence emission of PTA was observed, whereas other derivatives showed negligible effect.

**Figure 3.8.** The photograph of fluorescence color changes of PTA in water after the addition of 2 equivalents of representative purine bases (from left to right: PTA + adenine, PTA + guanine, PTA + UA, PTA + caffeine, PTA + theophylline, PTA + hypoxanthine).
Accordingly, no such binding was obtained from UV–vis and fluorescence titration experiments (Figure 3.9 and 3.10). This result demonstrates that chemosensor PTA exhibits high selectivity towards UA in water.

**Figure 3.9.** Fluorescence emission spectra (λ<sub>ex</sub> = 315 nm) of PTA (10 μM) upon addition of (a) adenine (100 μM), (b) guanine (100 μM), (c) caffeine (100 μM), (d) theophylline (100 μM), and (e) hypoxanthine (100 μM). (10 mM HEPES buffer, pH 7.4).
3.4.2.3. Determination of binding stoichiometry by Job’s plot

Judging from the fluorescence titrations, continuous variation method \(^{33}\) (Job’s plot) was used to determine the stoichiometric ratios of the host and the specific guest, which was found to be 1:2 for probe PTA with UA complexation (Figure 3.11).

**Figure 3.10.** Plot of fluorescence intensity of PTA (10 μM) vs. concentration of different guests (100 μM) at 377 nm in H₂O at pH 7.4

**Figure 3.11.** Job’s plot of PTA with UA in water, 10 mM HEPES buffer, pH 7.4, ([PTA] = [UA] = 100 μM) by fluorescence method, which indicates 1:2 stoichiometry.
3.4.2.4. Evaluation of the association constants for the formation of PTA–UA complex

By UV–vis method:
The substrate binding interaction was calculated according to the Benesi–Hildebrand equation.\(^{34}\)

\[
\frac{A_0}{A - A_0} = \left( \frac{\epsilon_0}{\epsilon_0 - \epsilon} \right)^2 \left( \frac{1}{K_B[\text{Substrate}]} + 1 \right) \quad \ldots (i)
\]

Here \(A_0\) is the absorbance of receptor in the absence of guest, \(A\) is the absorbance recorded in presence of added guest, \(\epsilon_0\) and \(\epsilon\) are the corresponding molar absorption co-efficient and \(K_B\) represents the substrate binding interaction with guest.

**Figure 3.12.** Linear regression analysis (1/[G] vs 1/\(\Delta I\)) for the calculation of association constant values by UV–vis titration method.

The association const. (\(K_a\)) of PTA for sensing UA was determined from the equation: \(K_a = \text{intercept}/\text{slope}\). From the linear fit graph (Figure 3.12) we get intercept = 3.66842, slope = 1.60096 \times 10^{-4}. Thus we get, \(K_a = 3.66842/(1.60096 \times 10^{-4}) = 2.30 \times 10^4\).

By fluorescence method:
Binding constant of the chemosensor PTA was also calculated through fluorescence emission method by using the equation (ii), where \(I_0, I_{\text{max}}, \text{ and } I\) represent the emission
intensity of free PTA, the maximum emission intensity observed in the presence of added UA at 377 nm ($\lambda_{ex} = 315$ nm), and the emission intensity at a certain concentration of UA added, respectively.

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

$[G]$ is the concentration of the guest UA. $[H]$ is the concentration of the host PTA.

![Graph](image)

**Figure 3.13.** Linear regression analysis ($1/[G]$ vs $1/\Delta I$) for the calculation of association constant values by fluorescence titration method.

The association const. ($K_a$) of PTA for sensing UA was determined from equation: $K_a = \text{intercept/slope}$. From the linear fit graph (Figure 3.13) we get intercept= $-0.00634$, slope = $2.04236 \times 10^{-7}$. Thus we get, $K_a = 0.00634/2.04236 = 3.10 \times 10^4$.

### 3.4.2.5. Calculation of limit of detection (LOD)

The detection limit of the receptors for UA 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 UA was measured by 10 times and the standard deviation of blank measurements was calculated. The limit of detection (LOD) of PTA for sensing UA was determined from the following equation: $\text{LOD} = K \times \text{SD}/S$.

Where $K = 2$ or 3 (we take 2 in this case); $\text{SD}$ is the standard deviation of the blank receptor solution; $S$ is the slope of the calibration curve.
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Figure 3.14. Linear fit curve of PTA at 377 nm with respect to UA concentration.

For PTA with UA: From the linear fit graph (Figure 3.14) we get slope = $6.11083 \times 10^6$, and SD value is 1.58283. Thus using the above formula we get the limit of detection = 0.518 µM. Therefore the chemosensor PTA can detect UA up to this very lower concentration.

### 3.4.2.6. pH titration study

From the pH titration experiment of the receptor PTA with UA we found that at biological pH i.e. at pH 7.4 the host–guest complexation occurs most prominently (Figure 3.15).

Figure 3.15. Change in fluorescence intensity of free chemosensor PTA (black) and in presence of 2 equiv. of UA in water (100 µM) (red) at $\lambda_{ex}$ 315 nm (10 mM HEPES buffer, pH 7.4) with different pH conditions.
3.4.2.7. $^1$H NMR titration study

In order to know more about interactions between UA and probe PTA, $^1$H NMR titration was also performed in D$_2$O (Figure 3.16). Both the amide proton of PTA, one from thymine moiety and another from thymine acetamide, displayed continuous down field shift with increasing addition of UA by 0.19 and 0.38 ppm, respectively. This observation indicates the presence of hydrogen bonding interaction between the amide linkages of UA with the complementary amide bond of probe PTA. In contrast, remaining hydrogens in the host were affected very small. These observations suggested that only amide proton of the probe participated to complex with UA.

![Figure 3.16. Partial $^1$H NMR titration [400 MHz] of PTA in D$_2$O at 25°C and the corresponding changes after the gradual addition of different equiv. of UA in D$_2$O from (a) only PTA, (b) PTA + 1 equiv. of UA, (c) PTA + 1.5 equiv. of UA, (d) PTA + 2 equiv. of UA; [inset] UA.](image)

3.4.2.8. DFT study

Binding of UA by PTA has also been investigated by quantum chemical calculations at the TDDFT level using the B3LYP/6-31+G(d,p) level of theory in presence of solvent water. Solvent effects were incorporated using COSMO solvent model$^{36}$ implemented in ORCA.$^{37}$
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The most favorable geometry of PTA–UA complex was found in Figure 3.17, which consists of five hydrogen bonds with bond distances around 1.36Å. The π electrons on the HOMO of PTA–UA complex was mainly located on the whole π-conjugated –NH–C=O framework including the pyrene moiety but the LUMO is mostly positioned at the interior of the guest UA. Figure 3.18 shows the energy optimized structure of PTA–UA complex.

**Figure 3.17.** Molecular orbitals (HOMO and LUMO) and electronic contribution of the relevant excitations of PTA (above) and PTA–UA complex (below)

**Figure 3.18.** The energy optimized structure of PTA–UA by TDDFT//B3LYP/6-31+G(d,p) + solv(COSMO) method.
Moreover, the HOMO–LUMO energy gap of the PTA–UA complex becomes smaller relative to that of PTA (Figure 3.19). The energy gaps between HOMO and LUMO in the PTA and PTA–UA complexes were calculated as 3.72 eV and 2.81 eV, respectively.

**Figure 3.19.** HOMO and LUMO distributions of PTA and the PTA–UA complex

### 3.4.2.9. Live cell imaging

The desirable features of the probe PTA such as high sensitivity with a “turn–on” fluorescence, fast response, reversibility, good performance at physiological pH, and high selectivity encouraged us to further evaluate the potential of the sensor for imaging UA in living cells. Vero cells (very thin endothelial cells) (Vero 76, ATCC No CRL–1587) were used as model.

**Figure 3.20.** Confocal microscopic images of the probe PTA in Vero 76 cells pretreated with UA. (a) Bright field image of the cells of controlled set. (b) Only PTA at 10 μM
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concentration, nuclei counterstained with DAPI (1 mg mL\(^{-1}\)). (c) Bright field image of the cells treated with PTA and UA (100 μM). (d) Image scan of probe PTA and UA (100 μM). All images were acquired with a 40X objective lens with the applied wavelengths: \(E_{\text{excitation}} = 350\text{ nm}, E_{\text{emission}} = 470\text{ nm}\).

Vero cells incubated with the chemosensor PTA exhibited extremely weak fluorescence, whereas a clear blue fluorescence signal was observed in the cells stained with both the fluorophore PTA and UA, which in good agreement with the fluorescence “turn–on” profile of the sensor in presence of UA in the solution [Figure 3.20 (a–d)]. To the best of our knowledge, this is the first known pyrene based substituted thymine conjugate which has been demonstrated to recognize UA in live cells.

3.4.2.10. Cytotoxicity assay

Cells were intact and showed a healthy spread and adherent morphology during and after the labelling process with sensor PTA, indicating absence of cytotoxic effects. However, to materialize this objective, it is a prerequisite to assess the cytotoxic effect of probe PTA, UA and the complex on live cells. The well-established MTT assay, which is based on mitochondrial dehydrogenase activity of viable cells were adopted to study cytotoxicity of above mentioned compounds at varying concentrations. Cytotoxicity measurements for each experiment shows that both probe PTA and PTA–UA complex do not exert any adverse effect on cell viability\(^38\) (Figure 3.21).

![Figure 3.21. MTT assay to determine the cytotoxic effect of PTA and PTA–UA complex on Vero 76 cells](image-url)
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Vero cells were incubated in PBS buffer (pH 7.4) containing 10 mM of the probe PTA for 20 min at 37°C, followed by washing the cells with the same buffer to remove the excess of the probes. At this stage, the confocal microscopy images of Vero cells displayed very weak intracellular fluorescence. However, upon the treatment of exogenous UA into the cells for 20 min at 37°C, the cells exhibited highly intense blue fluorescence. The control experiments carried out with PTA solution alone do not show any fluorescence (Figure 3.21). Thus, Vero cells incubated with PTA in the presence of UA showed much greater fluorescence emission as compared to the cells which were not incubated. This suggests that UA is responsible for enhancing the fluorescence of PTA in the cells. These results clearly indicate that the chemosensor PTA is an effective intracellular UA imaging agent.

3.5. Conclusion

In conclusion, we have successfully developed the pyrene appended thymine derivative PTA as a promising chemosensor for the detection of UA in water with very low concentration. The chemosensor PTA exhibits a highly sensitive and selective fluorescence enhancement towards UA over other biologically important purine derivatives by producing “turn–on” fluorescence at 377 nm in aqueous buffer (pH 7.4). The host–guest binding mechanism was demonstrated on the basis of absorption, fluorescence, 1H NMR titration and TDDFT calculations which provide information about the formation of 1:2 complexes between PTA and UA. The association constant (K_a) for the complexation between PTA with UA is in the order of 10^4 M^-1 based on spectroscopic studies, suggesting a strong binding with UA. The probe has been shown to be sensitive with a minimum detection limit of 0.5 µM in water at neutral pH. The selective “turn–on” detection of UA in water was successfully expressed in live cells for the first time. The chemosensor PTA does not show any toxicity on the tested cells and PTA–UA complex does not exert any significant adverse effect on cell viability. In vitro studies established the ability of the chemosensor PTA to detect UA in biological systems with excellent selectivity. PTA is an obvious choice for cellular imaging study due to the presence of pyrene appended two amide linkages which help to achieve the strongest complementary binding in case of UA among other purine derivatives. The above observations demonstrate the potential efficacy of the chemosensor PTA as a molecular probe in biological systems.
3.6. Experimental section

Materials and methods

1-pyrenemethylamine hydrochloride, thymine, bromoacetyl chloride and uric acid 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 D$_2$O was 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. FTIR spectra was recorded as KBr pellets using a SHIMADZU FTIR–8400S 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; q= quartet; m = multiplet.

Synthetic procedures

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 dropwise 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.

**PTA:** To a solution of anhydrous K$_2$CO$_3$ (2.5 g, 18 mmol) in dry DMF was added thymine (0.2 g, 1.50 mmol). The mixture was stirred for 0.5 h. Then compound 1 (0.85 g, 2.5 mmol) was added to the solution and stirred for 48 h. Then, the reaction mixture was poured into water. The solution was extracted with EtOAc (3 × 50 mL), and the combined organic layer was washed with 5% aqueous HCl (50 mL), 10% aqueous Na$_2$CO$_3$ (50 mL) and finally with water and then dried over by anhydrous MgSO$_4$. After removing the solvents, the residue was chromatographed on silica gel with Ethyl acetate/methanol= 8:1 v/v as eluent to give 0.05 g (23%) of compound 1 as brown solid. $^1$H NMR (DMSO-d$_6$, 400 MHz): δ (ppm) 1.78 (s, 3H), 4.41 (s, 2H), 5.05 (d, J = 8 Hz, 2H), 7.51 (s, 1H), 8.07–8.38 (m, 10H), 8.92 (t, J = 8 Hz, 1H). $^{13}$C–NMR (DMSO-d$_6$, 400 MHz): δ (ppm) 12.87, 41.45, 50.50, 63.75, 108.93, 124.08, 124.84, 124.94, 125.65, 126.12, 126.22, 127.21, 127.56, 128.01, 128.32, 128.55, 131.09, 131.25, 131.73, 133.35, 143.25, 152.05, 165.63, 167.94. FTIR (cm$^{-1}$): 3160.98 (N–H str.), 2370.35 (C=C), 1695.31 (C=O str.), 2910.68 (sp$^3$-CH$_3$). HRMS (m/z, %): 420.2314 [(M + Na$^+$), 100 %]; Calculated for C$_{24}$H$_{19}$N$_3$O$_3$: 397.4271. Anal. Calcd. for C$_{24}$H$_{19}$N$_3$O$_3$: C, 72.55; H, 4.79; N, 10.57; O, 12.09; Found: C, 72.58; H, 4.77; N, 10.59; O, 12.06.

**Preparation of test solution for UV–vis and fluorescence study**

A stock solution of probe PTA (10 μM) was prepared in water. An UA solution of concentration 100 μM was prepared in Millipore water. All experiments were carried out in water (10 mM HEPES buffer, pH 7.4). During titration, each time a 10 μM solution of PTA was filled in a quartz optical cell of 1 cm optical path length and UA 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 UA. For fluorescence measurements of PTA, excitation was provided at 315 nm, and emission was collected from 360 to 460 nm.

**General procedure for Job’s plot**

The stoichiometry was determined by the continuous variation method (Job’s plot). In this method, solutions of host (PTA) and guest (UA) of equal concentrations (10 μM) were
prepared in the solvents used in the experiment. Then host and guest solutions were mixed in different proportions maintaining a total volume of 3 mL of the mixture. All the prepared solutions were kept for 1 h with occasional shaking at room temperature. Then emissions of the solutions of different compositions were recorded and plotted with different sets of volume ratio of host and guest ($\lambda_{ex}= 315$ nm, $\lambda_{em}= 377$ nm).

**Computational details**

Geometries have been optimized using the B3LYP/Def2SVP level of theory in presence of solvent water. Solvent effects were incorporated using COSMO solvent model$^{31}$ implemented in ORCA.$^{32}$ 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.$^{32}$

**Live cell imaging**

**Cell line and cell culture:** Vero cell (Vero 76, ATCC No CRL–1587) 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 $\mu$g/mL), and streptomycin (100 $\mu$g/mL). The Vero 76 were obtained from the American Type Culture Collection (Rockville, MD) and maintained in DMEM containing 10% (v/v) fetal bovine serum and antibiotics in a CO$_2$ incubator. 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 $\times$ 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 $\times$ 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), and then treated according to the experimental need.

**Cell imaging study:** For confocal imaging studies Vero cells, $1 \times 10^4$ cells in 1000 $\mu$L of medium, were seeded on sterile 35 mm $\mu$-Dish, glass bottom culture dish (ibidi GmbH,
Germany), and incubated at 37°C in a CO₂ incubator for 10 hrs. Then cells were washed with 500 µL DMEM followed by incubation with 1.0 × 10⁻⁴ M UA dissolved in 500 µL DMEM at 37°C for 1 hr in a CO₂ incubator and 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 Olympus Fluoview (version 3.1a; Tokyo, Japan) with excitation at 285 nm and 312 nm monochromatic laser beam, and emission spectra were integrated at over the range 300–450 nm (single channel). The cells were again washed thrice with phosphate buffered saline PBS (pH 7.4) to remove any free UA and incubated in PBS containing probes PTA to a final concentrations of 10⁻⁵ M, incubated for 10 min followed by washing with PBS three times to remove excess probe outside the cells and images were captured. According to the need of the experiment we follow similar procedures to label the cell nuclei by treatment with DAPI (1 µg/mL) followed by three times wash with PBS and subsequently image was captured with excitation wavelength of laser was 350 nm, and emission was 470 nm. 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.

**Cytotoxicity assay**

The cytotoxic effects of probe PTA and PTA–UA complex were determined by an MTT assay following the manufacturer’s instruction (MTT 2003, Sigma–Aldrich, MO). Vero cells were cultured into 96–well plates (10⁴ cells per well) for 24 h. After overnight incubation, the medium was removed and various concentrations of PTA and PTA–UA complex (0, 5, 25, 50, 75 and 100 µM) made in DMEM were added to the cells and incubated for 24 h. Control experiments were set with DMSO, cells without any treatment and cell–free medium were also included in the study. Following incubation, the growth medium was removed and fresh DMEM containing MTT solution was added. The plate was incubated for 3–4 h at 37°C. Subsequently, the supernatant was removed, the insoluble colored formazan product was solubilized in DMSO, and its absorbance was measured in a microplate reader (Perkin–Elmer) at 570 nm. The assay was performed in triplicate for each concentration of PTA and PTA–UA. The cell viability was calculated by the following formula: (mean OD in treated wells / mean OD in control wells) × 100.
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3.7. References

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**Publication from this chapter:**

Appendix
Supporting information:

**Figure S.3.1.** $^1$H NMR of compound 1 in DMSO-$d_6$ (400 MHz)

**Figure S.3.2.** $^{13}$C NMR of compound 1 in DMSO-$d_6$ (400 MHz)
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**Figure S.3.3.** $^1$H NMR of PTA in DMSO-d$_6$ (400 MHz)

**Figure S.3.4.** $^{13}$C NMR of PTA in DMSO-d$_6$ (400 MHz)
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Figure S.3.5. FTIR Spectrum of PTA

Figure S.3.6. HRMS of PTA
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Competitive absorbance study:

![UV–vis absorption spectra](image)

**Figure S.3.7.** UV–vis absorption spectra of PTA (10 μM) upon addition of (a) adenine (100 μM), (b) guanine (100 μM), (c) caffeine (100 μM), (d) theophylline (100 μM), and (e) hypoxanthine (100 μM) (10 mM HEPES buffer, pH 7.4)
Figure S.3.8. Changes of relative absorption intensity (A/A₀) of PTA (0.1 μM) as a function of [UA]/[PTA] in water at neutral pH.

Figure S.3.9. Fluorescence intensity changes of PTA (0.1 μM) upon addition of various concentrations of UA in water at neutral pH (λₑₓ = 315 nm).
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**DFT Study:**

**Table S.3.1.** 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/6-31+G(d,p) + solv(COSMO) based on the optimized ground state geometries.

<table>
<thead>
<tr>
<th>Molecules</th>
<th>Electronic Transition</th>
<th>Excitation Energy$^a$</th>
<th>f$^b$</th>
<th>Composition$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>UA</td>
<td>$S_0 \rightarrow S_1$</td>
<td>4.861 eV 255.1 nm</td>
<td>0.1905</td>
<td>$H \rightarrow L$</td>
</tr>
<tr>
<td></td>
<td>$S_0 \rightarrow S_3$</td>
<td>5.373 eV 230.8 nm</td>
<td>0.2038</td>
<td>$H \rightarrow L + 1$</td>
</tr>
<tr>
<td></td>
<td>$S_0 \rightarrow S_{12}$</td>
<td>7.161 eV 173.1 nm</td>
<td>0.3764</td>
<td>$H-4 \rightarrow L$</td>
</tr>
<tr>
<td>PTA</td>
<td>$S_0 \rightarrow S_2$</td>
<td>3.793 eV 326.9 nm</td>
<td>0.3485</td>
<td>$H \rightarrow L$</td>
</tr>
<tr>
<td></td>
<td>$S_0 \rightarrow S_8$</td>
<td>4.788 eV 259.0 nm</td>
<td>0.4216</td>
<td>$H-2 \rightarrow L$</td>
</tr>
<tr>
<td>PTA-UA</td>
<td>$S_0 \rightarrow S_4$</td>
<td>3.758 eV 329.9 nm</td>
<td>0.2459</td>
<td>$H \rightarrow L$</td>
</tr>
<tr>
<td></td>
<td>$S_0 \rightarrow S_{18}$</td>
<td>4.750 eV 261.0 nm</td>
<td>0.2808</td>
<td>$H \rightarrow L + 2$ $\text{H-4} \rightarrow L$</td>
</tr>
</tbody>
</table>

[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 S.3.2.** Energies of the highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO)

<table>
<thead>
<tr>
<th>Species</th>
<th>$E_{\text{HOMO}}$ (a.u)</th>
<th>$E_{\text{LUMO}}$ (a.u)</th>
<th>$\Delta E$ (a.u)</th>
<th>$\Delta E$ (eV)</th>
<th>$\Delta E$ (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UA</td>
<td>-0.209224</td>
<td>-0.024793</td>
<td>0.184431</td>
<td>5.01862571</td>
<td>115.7322046</td>
</tr>
<tr>
<td>PTA</td>
<td>-0.20384</td>
<td>-0.066943</td>
<td>0.136897</td>
<td>3.72515902</td>
<td>85.90416802</td>
</tr>
<tr>
<td>PTA-UA</td>
<td>-0.201718</td>
<td>-0.09682</td>
<td>0.104898</td>
<td>2.81442017</td>
<td>65.82446244</td>
</tr>
</tbody>
</table>