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Differential detection and quantification of cyclic AMP and other adenosine phosphates in live cell

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A new naphthol-based rhodamine derivative (NpRD) has been developed for selective and differential detection of adenosine 3', 5'-cyclic monophosphate (cAMP) and adenosine phosphates (APs) (ATP, ADP, AMP) from other nucleotides.The simple detection and quantificatioin of cAMP in human blood cell and in other samplesby 'turn on' fluorescent property of this chemosensor through colorimetry or fluorometry makes it unique for probable usages in high throughput screening.

Nucleotides are indispensable constituents of all living cells. They make up DNA, RNA and serve as important energy sources. Few of them also have key roles as signaling molecules that contribute to the regulation of cellular pathways in all form of life. The most important of such nucleotides is adenosine 3', 5'-cyclic monophosphate (**cAMP**).[1,2] **cAMP** is synthesized from ATP by adenylatecyclase present on the inner side of the plasma membrane. It regulates a striking number of physiological processes, including intermediary metabolism, cellular proliferation and neuronal signaling by altering basic pattern of gene expression. It also plays an important role in cell activation, synaptic transmission, exocrine and endocrine secretion.[3-5] **cAMP** driven mechanisms are central to the pathogenesis and also well established as a potential regulator of innate and adaptive immune cell functions.[6] Knowledge of the biological role of **cAMP** has not been widely applied clinically; sufficient acquaintance has now accumulated to make research in this area affluent and desirable.

Though it is well known fact that the cellular concentration of **cAMP** is the key player behind above mentioned physiological changes[7], till date no standard direct detection method of **cAMP** has been explored in live cell using any chemical sensor. It is much demanding to develop a reliable analytical method for the detection and quantification of **cAMP**. Among several detection strategies, fluorescent techniques are most attractive and have been widely applied in the detection of biologically relevant ions and bio-molecules in recent years[8] mainly due to their high sensitivity, cost effective, easy implementation, and real-time detection.[9,10] It has a substantial importance to design and synthesize fluorescent probes[11] with desirable properties due to their fundamental role in medical, environmental and biological applications.[12,13] The currently available biosensors for **cAMP** are based on the targets, including **cAMP**-dependent protein kinase (PKA), cyclic nucleotide gated channels (CNGCs) and exchange protein directly activated by **cAMP** (Epac). These biosensors are fluorescently tagged molecules that use FRET or BRET to determine changes in **cAMP** levels, or Ca^{2+} specific dyes that indirectly measure **cAMP**. The choice of fluorescent probe which will label these biomolecules is the main concern. The matches between the donor and receptor also limit their usage in live cell.[14]Further improvement in this field is only limited with the detection limit and enhancement of the FRET sensors. Moreover, the difficulties in basic methodology like FRET or BRET lies in the expression of fluorescent-tagged biomolecules. It is of course a tedious experiment to constructa recombinant plasmid clone, its delivery, level of its expression inside cell, level of its binding to specific target and crosstalk with other cellular factors and can limit their application.[14] Within the limited **cAMP** biosensors in hand, the choice of a particular type is very important for specific application. Each of these has a strength and weakness, which in turn might be affected by the particular issue under consideration, the availability of experimental skills or access to specialized equipment. In nutshell, choosing a biosensoralways depends on the consideration like ease of application, kinetic behavior, target to **cAMP**, cellular effects on the probe and suitability for high-throughput screening. A chemosensor, in other hand, is thus far in front than the biosensor to easily detect **cAMP** in biological sample and in solution, in single experiment or in high-throughput screening, in much cost effective and selective manner (performance comparison shown in table S1).

Here, we report a differential and selective recognition and determination of **cAMP** along with APs from other nucleotides (Fig. S1) by a novel naphthol-based rhodamine derivative (**NpRD**) through its 'turn on' fluorescence property. Rhodamine derivative is one of the best choice chemosensor because of its spirolactam ring which showed no fluorescence,

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but when the ring opened, the amide form showed strong fluorescence emission and UV-visible absorbance. Rhodamine derivatives are widely used as potential fluorophore as they generate fluorescence and visible colorimetric response in presence of a suitable guest.[15]

NpRD was synthesized from a simple reaction of rhodamine B and ethylenediamine, followed by reaction with 2-naphthol resulting 80% overall yield. The structure of **NpRD** was confirmed by ^{1}H NMR, ^{13}C NMR, IR, MS, and X-ray analysis (Fig.S2-S6).The crystal structure (Fig.1) clearly represents the unique spirolactam ring formation (H4···O2 2.09Å, N4···O2 2.880(5)Å, angle N4-H4···O2 153°). Two planes of the spiro form of rhodamine framework are coordinated in a mutually vertical position.

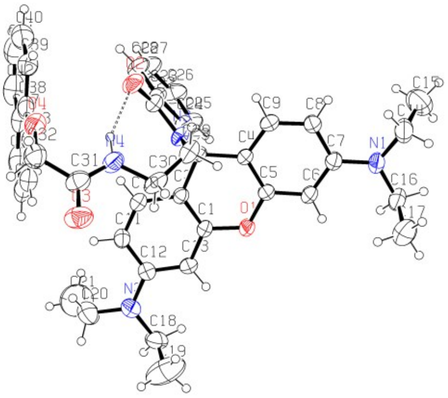
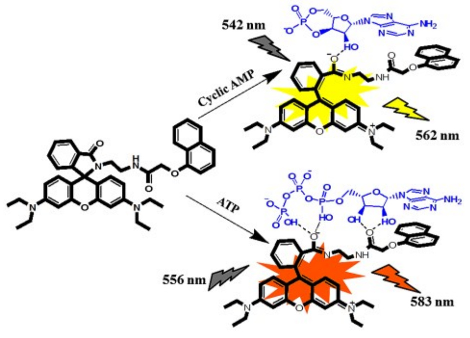


Fig. 1: ORTEP diagram of probe **NpRD**. Ellipsoids are drawn at 50% probability level.

As far as we aware, this is the first report, where we envisage the possibility of using a rhodamine derivative for the *in vitro* and *in vivo* detection and quantification of **cAMP** by colorimetric and fluorimetric method through anion recognition mechanism, based on the change in structure from spiro cyclic to open cycle form. Probe **NpRD** strongly responses a selective colorimetric and fluorimetric change only upon addition of **cAMP**(upto 6 equivalent) and other adenosine



Scheme 1: Fluorescence changing of **NpRD** on addition of **cAMP** and ATP

phosphates (Scheme 1). This indicates that the probe undergoes irreversible spirolactam ring opening via coordination of **cAMP** or other APs and shows selectivity with suitable detection range.

There may be two main reaction sites that cause the spirolactam ring to be opened cooperatively. One is a hydrogen bond interaction between the amide moieties of the probe and sugar and/or phosphate groups of APs; the other is a π-π stacking interaction between the naphthalene moiety of the probe and adenine group of **cAMP** or APs. The stronger hydrogen bond interaction depends on the number of phosphate groups present in APs which is likely to be the reason for the differential selectivity of the probe to **cAMP** and APs over other nucleotides. Moreover, the π-π stacking interaction between the probe and adenine group plays a significant role in the sensing system, which accounts for the selective and differential detection of the probe to **cAMP** or ATP over GTP, GMP, CTP, UTP, TTP, inorganic phosphates and other anions. The π-π stacking interaction between the probe and adenine is more effective due to well matched spatial orientations. Hence, the combination of two different reaction sites upholds the probe for possessing high selectivity to APs over other nucleotides and biological anions.

To ensure the interaction of **NpRD** with **cAMP**, UV-vis and fluorescence titration were carried out by gradual addition of 100 µM of **cAMP** to a fixed concentration (20 µM) of **NpRD** in CH_3CN/H_2O (1:6, v/v) (Fig. S7). In the presence of **cAMP**, the probe gave rapid responses, yielding full signal in 2-3 minutes with an absorption at 542 nm and an emission maxima at 562 nm (Fig.2) with 40-fold increase in fluorescence (K_a = 7.8 × $10^{3}M^{-1}$, see ESI†). The binding stoichiometry of probe **NpRD** with **cAMP**is 1:1 and the binding constant[16] of **NpRD-cAMP** complex as obtained from the UV-vis titration is K_a= 2.5 × $10^{3}M^{-1}$ which is 4 times lower than that of ATP (K_a= 1.09 ×

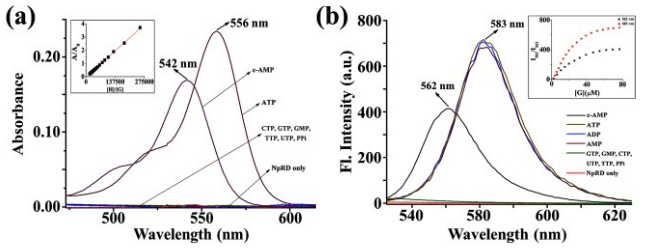


Fig. 2. (a) Comparative UV-vis absorption spectra of **NpRD** (c = 2 × 10^{-5} M) in CH_3CN/H_2O, 1:6 (v/v), (pH 7.0, 10mM phosphate buffer) after addition of **cAMP**, APs and other biological phosphates upto 6 equiv.(Inset) binding const. plot of **NpRD** with **cAMP**. (b) Comparative fluorescence emission spectra of **NpRD** (c = 2 × 10^{-5} M) in CH_3CN/H_2O (1:6, v/v) after addition of 6 equiv. of cAMP, APs and other biological phophates (λ_{ex} = 485nm). (Inset)Comparison plot of fluorescence intensity of **cAMP** (black points) and ATP (red points) with fixed concentration of **NpRD**.

$10^{4}M^{-1}$) (Fig. S8 and S9).It was found that **NpRD** has a detection limit of 2.8 µM for **cAMP** (Fig. S10). The continuous variation method[17] was used to validate the above said stoichiometry (Fig. S11).

Fig. 2b shows fluorescence spectra (λ_{ex} = 485 nm) of **NpRD** (20 µM) with respective biological phosphate anions (upto 6 equiv). Without such anions, **NpRD** showed no noticeable fluorescence signal, however addition of **cAMP** created a remarkably enhanced (40-fold) and blue-shifted (21 nm) yellow fluorescence at 562 nm. APs also persuade a large emission enhancement (70-fold) at 583 nm (orange fluorescence). This implies that **NpRD** could be utilized as a selective and differential sensor to measure the level of **cAMP** and APs within a minute.

The colorimetric detection was performed to understand the binding potencies and sensing selectivity of **NpRD** with different biological phosphates (**cAMP**, ATP, ADP, AMP, GTP,

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UTP, TTP and PPi) and other anions (Fig. S12). Fig. 3 displays the nature of fluorescence of the complexes upon addition of various guests with naked eye. The colorless clear solution of

Fig. 3. Photograph of visual (upper panel) and fluorescence (lower panel) color changes of **NpRD**(c= 2 × 10^{-5} M) in CH_3CN/H_2O (1:6, v/v) (pH 7.0) after the independent addition of 6 equivalents of various nucleotides and biological phosphates in 10^{-4} M concentration.

the **NpRD** immediately turned into light pink after addition of **cAMP** while **AMP**, **ADP** and **ATP** turned the solution into dark pink. However the addition of the other nucleotides to **NpRD** did not show any color change of the solution. The similar experiment has been performed under UV lamp where **cAMP** showed a strong yellow fluorescence and ATP, ADP, AMP showed strong orange fluorescence. This result demonstrated that probe **NpRD** exhibited high selectivity towards **cAMP** and other APs over other nucleotidesin acetonitrile-water medium (1:6, v/v) at neutral pH.

In order to know more about interactions of **NpRD** with **cAMP**, ^{1}H NMR titration was also executed in DMSO-d_6 (Fig. S13). The aromatic protons of probe **NpRD** shifted little bit up field and became broader upon sequential addition of further **cAMP**. This is due to the decrease in electron density of the aromatic ring, indicating that probe **NpRD** actually coordinates with **cAMP** and electrons are withdrawn by hydroxyl group of **cAMP**. But the –CH_2 peak of ethylenediamine moiety at 3.16 ppm displayed a little downfield shift with gradual addition of **cAMP** and finally got lapsed. This coordination implies the electron drifting from rhodamine moiety to **cAMP** through the amide carbonyl groups of **NpRD**. In ^{13}C NMR titration, the aromatic region becamelittle downfield compared to the original one (Fig. S14).The spiro cycle carbon peak at 68 ppm is shifted to 142 ppm, indicates the opening of spiro ring. The major participation of amide moiety with the guest has also been evident from IR titration (Fig. S15).

Interaction of **cAMP** and ATP with **NpRD** has further been

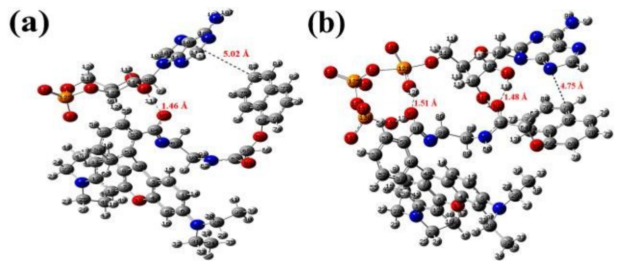

Fig.4. Optimized structures of **NpRD** with (a) **cAMP** and (b) ATP. The hydrogen bond interactions and the π-π stacking interaction are denoted by dotted line.

comprehended by quantum chemical calculations at the DFT level with the B3LYP/6-31+G (d,p) method using the Gaussian 09 program. The optimized structures signify the most favorable geometry of **NpRD-cAMP** and **NpRD**-ATP complex.

In the energy minimized structures (Fig. 4) it has been seen that the OH groups of the phosphate moiety in ATP interact with the lactam oxygen of the rhodamine moiety and the sugar hydroxyl groups bind other amide oxygen of **NpRD** by hydrogen bonds (the distance is 1.51 and 1.48 Å, respectively). However in case of **cAMP**, only sugar hydroxyl group interact with lactam oxygen (distance is 1.46 Å) of the rhodamine. The well-matched spatial orientations between the ATP (or APs) and **NpRD** play an important role while adenine come closer and in parallel to naphthol (the distance is 4.75 Å) to achieve minimized energy. The orientation between **cAMP** and **NpRD** make adenine a little bit tilted than reasonable parallel fashion to naphthol (the distance is 5.02 Å). Above observations correlate the absorbance and fluorescence properties of **NpRD**-APs complexes. The stronger π-π stacking interactions between the adenine group of APs and naphthol group of **NpRD** make the complex much stronger. In contrast, the reaction pattern between **NpRD** with other nucleotides was studied which show no π-π stacking interaction among them on account of their mismatched spatial configuration.

Furthermore, the specific and selective detection of **cAMP** by the probe **NpRD** was also monitored in biological sample. From the MTT assay[18] it has been found that cells were proliferating without any impairment when treated with probe **NpRD**, indicating absence of its cytotoxic effect (Fig. 5a) in working concentration. Although the treatment of cells with higher concentration of **cAMP** showed partial toxicity as it can alter several cellular physiology, but the concentration of **cAMP** used for cell imaging studies does not significantly decrease cell proliferation.

To demonstrate the desirable features of the probe with ‘turn on’ fluorescence at physiological pH, we further evaluated the potential of the sensor for detecting **cAMP** and APs (Fig. 5b and S16) in live cells through confocal microscopy.Hep-2 cells (Human cell ATCC: CCL-23) incubated with probe **NpRD** exhibited basal level fluorescence for the presence of indigenous **cAMP**, whereas a strong blue fluorescence signal was observed in the cells treated with additional **cAMP**, which is in corroboration with that of the in-solution observation.

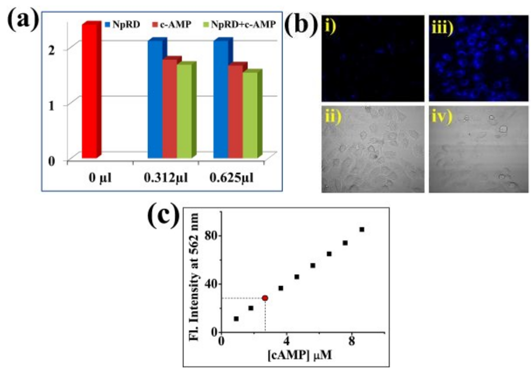

Fig. 5. (a)MTT assay diagram shows the cellular proliferation in presence of **NpRD** and **cAMP**. (b) Confocal microscopic images of Hep-2 cells (i) with **NpRD** treatment, (iii) with **NpRD**and exogenous**cAMP** (c = 1 × 10^{-4} M) treatment, (ii) and (iv) are the bright-field images of (i) and (iii). All images were acquired with a 40X objective lens with the applied wavelengths: $E_{excitation}$=555 nm, $E_{emission}$=565 nm. (c) Estimation of unknown concentration of **cAMP** (red point) collected from human blood sample using the standard curve.

The concentration of **cAMP** was also quantified from human blood sample collected from P. M. Hospital, Visva-Bharati University. 4 ml human blood of normal healthy person was centrifuged to collect the blood cells. Blood cells were dissolved with 10 mM Tris-Cl buffer (pH 7.0) and lysed by osmotic shock with ice cold water. Supernatant were collected after centrifugation and analyzed to detect **cAMP** present in this sample with the help of **cAMP** standard fluorescence curve (Fig.5c and S17) using the selective detection ability of the probe **NpRD**. All estimation has been done in triplicate.From the standard curve it has been found that the concenctration of **cAMP** in the trested blood samples are 2.8 μM.To authenticate the above result the real sample was spiked with known concentration of **cAMP** and used for sensing purposes by adding known volumes from each sample to the **NpRD**. The recovery of the spiked samples was estimated to be in the range of 96‑98% (Table S2, ESI†).We further validated assay of **cAMP** from multiple human blood samples using **NpRD**. Each sample has been assayed in triplicate. The signal to noise ratio of each independent reading is between 16 and 21 (Table S3). The fold increase of fluorescence signals have also been statistically validated after calculating the Z' value for each set of measurement.[19] It has been found that in all tested samples the Z' score is more than 0.9, indicating an optimized and validated assay of **cAMP**.

In conclusion, we have successfully developed an unique naphthol-based rhodamine derivative **NpRD** for the selective and differential detection of **cAMP** and other adenosine phosphates in aqueous medium with very low concentration. **NpRD** exhibits highly sensitive and selective response differently towards **cAMP** and other APs both in the colorimetric and fluorimetric detection technique. This is the first known method where an anion sensing mechanism is based on the change in structure from spiro cyclic to open rhodamine form to make the probe fluorescent. The 'turn on' fluorescence for the selective detection of **cAMP** was successfully demonstrated in live cell and in human blood sample. This proves the potentiality of probe **NpRD** useful for investigating the presence of **cAMP** in biological systems.Our newly designed fluorescence chemosensor that specifically binds **cAMP** with detection limit in micromolar range makes it appropriate to use in diagnostics as well as in live cell imaging. The simple detection of **cAMP** by this chemosensor only through colorimetry or fluorometry makes it unique for probable usage in high throughput screening for mankind over existing biosensors which estimates **cAMP** indirectly.

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