Zn(II) based colorimetric sensor for ATP and its use as a viable staining agent in pure aqueous media of pH 7.2†

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Selective colorimetric detection of ATP in physiological conditions by a Zn(II)-based receptor is reported. This reagent was found to be non-toxic to the living cells and could be used for studying the growth of the yeast cells.

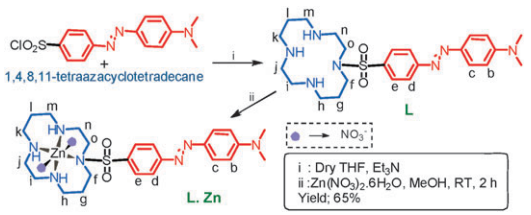
Phosphates are among the most significant anions in living cells and play crucial roles in numerous biological processes; like universal energy storage, ion-channel regulation, intercellular signalling mediation, protein phosphorylation, enzymatic reactions, DNA replication, *etc.*[1–4] Among these, adenosine-5′-triphosphate (ATP) is the one which is studied most due to its role as a molecular unit of currency for intercellular energy transfer.[2] The production of energy is achieved through the cleavage of one or two phosphate groups from ATP resulting in adenosine-5′-diphosphate (ADP) and adenosine-5′-monophosphate (AMP), respectively. Pyrophosphate (PPi) is also the product of ATP hydrolysis under cellular conditions.[3] More importantly, deficiency in ATP results in ischemia, Parkinson's disease and hypoglycaemia.[4] Thus, the monitoring of the ATP concentration level is crucial for the study of different cellular mechanisms, enzymatic processes and even cell apoptosis. Though, there are many examples of artificial receptors that are efficient in recognition of phosphates, reports on their bioanalytical application in physiological conditions are rather limited.[5–8] Such reports on specific recognition of ATP and its probable *in vivo* application are extremely rare and this led us to develop a reagent that is non-toxic to the living cells.[7,8]

The most widely studied receptor fragment that has been explored to date for recognition of ATP and other biological phosphates is derived from Zn(II)-complex of 2,2′-dipicolyl amine (dpa).[7,8] Among various Zn(II)-dpa-based receptors,[6,7] including ours,[8] use of a certain amount of non-aqueous solvent is essential in most cases due to the limited solubility of these receptors in water. As a part of our ongoing effort to develop a sensor molecule that is soluble in pure water and can be used for *in vivo* detection of ATP in living cells, we have synthesized a new Zn(II)-complex using a 1,4,8,11-tetraazacyclotetradecane derivative. We report herein its synthesis, characterization, specificity and relative binding affinity towards ATP as compared to other biologically important phosphates like CTP, ADP, AMP, PPi and phosphate.‡ Also, we have discussed the possibility of using this reagent as a viable staining agent for cell growth studies.

The intermediate ligand (*E*)-4-((4-(1,4,8,11-tetraazacyclotetradecan-1-ylsulfonyl)phenyl)diazenyl)-*N*,*N*-dimethylaniline (**L**) was synthesized by reacting 1,4,8,11-tetraazacyclotetradecane with (*E*)-4-((4-(dimethylamino)phenyl)diazenyl)benzene-1-sulfonyl chloride (Scheme 1).† This was purified prior to further use for reaction with $Zn(NO_3)_2{\cdot}6H_2O$ for synthesis of the corresponding Zn(II)-complex (**L.Zn**).†

Uv-vis spectra recorded for **L** and **L.Zn** (Fig. 1) show a broad absorption band with λ_{max} at 422 and 453 nm, respectively in CH_3OH–CH_3CN medium (3 : 7, v/v). A band maximum at 422 nm for **L** was attributed to the intercomponent (π–π^*) charge transfer (CT) band. This CT transition was further red shifted on coordination to the cationic Zn(II)-centre in **L.Zn**.† This CT band for **L.Zn** in 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer medium appeared at 463 nm.

In order to examine the binding behaviour of the reagent, **L.Zn** towards various anionic analytes, like ATP, CTP (cytidine triphosphate), ADP, AMP, PPi, $H_2PO_4^-$, SO_4^{2-}, $CH_3CO_2^-$, I^-, Br^-, Cl^-, F^-, CN^-, SCN^-, NO_3^- and NO_2^-, Uv-vis spectra for **L.Zn** were recorded in absence and presence of large excess (2000 mole equivalent) of respective anions in 10 mM HEPES buffer medium (pH 7.2). As shown in Fig. 1, a red shifted CT absorption band for **L.Zn** with a maximum at 503 nm was observed on addition of excess sodium salt of ATP. An insignificant shift of 9 nm was observed when CTP of comparable concentration was added (Fig. 1A). In contrast, the change for ADP was even less significant and other anionic analytes failed to induce any detectable change in electronic spectra (Fig. 1A). A visually detectable change in solution colour was observed on addition of ATP to **L.Zn** solution (10 mM HEPES buffer medium), whereas this change was barely detectable for CTP and ADP. For all other anionic analytes used in this study, solution colour remained unchanged. No change in spectra on addition of these anions suggests either no or a very weak binding of these anions to the Zn(II)-centre of **L.Zn**. Systematic spectrophotometric



Scheme 1 Methodology adopted for synthesis of **L** and **L.Zn**.

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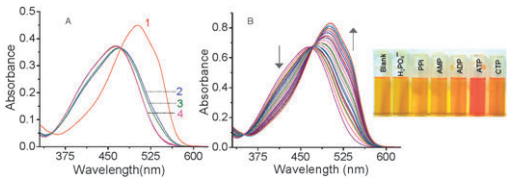

Fig. 1 (A) Absorption spectra of **L.Zn** (5.32 μM) in presence of various anions (~10.6 mM); 1: ATP; 2: CTP; 3: ADP; 4: blank, F^-, Cl^-, Br^-, I^-, CH_3COO^-, $H_2PO_4^-$, PPi, SO_4^{2-}, NO_3^-, NO_2^-, CN^-, AMP, SCN^-. (B) Absorption spectra of **L.Zn** (9.46 μM) in presence of varying [ATP] (0–17.28 mM). Inset: represents change in colour of **L.Zn** (8.87 μM) in absence and presence of various phosphate ions. From left to right: Blank, with added $H_2PO_4^-$, PPi, AMP, ADP, ATP, CTP (anion concentration ~500 μM). For all experiments pH of 7.2 was maintained (10 mM HEPES buffer solution) at 25 °C.

titrations were carried out in 10 mM HEPES buffer medium in order to evaluate the relative binding affinities of ATP (Fig. 1B), CTP and ADP towards **L.Zn** and respective binding constants were found to be 978 ± 4 M^{-1}, 220 ± 15 M^{-1} and 142 ± 15 M^{-1} at 25 °C.† The binding constant was evaluated based on the absorbance changes at 503 nm.

Binding of ATP with **L.Zn** was also confirmed by ^{31}P NMR spectral studies. Upfield shifts for the ^{31}P signals for the α- (0.07 ppm), β- (0.09 ppm) and γ- (0.05 ppm) phosphorus atoms of the ATP bound to **L.Zn** were observed (Fig. 2). The shifts in ^{31}P NMR signals for α-, β- and γ-P atoms signify the binding to Zn-atom of **L.Zn** through an oxygen atom bearing the negative charge of the respective phosphate unit. Relatively weaker interaction of the $O_{[\gamma\text{-}PO_4]}^-$ and $O_{[\alpha\text{-}PO_4]}^-$ relative to the $O_{[\beta\text{-}PO_4]}^-$ unit accounts for the smaller $\Delta\delta$ shift in ^{31}P NMR spectra. This observation also confirms the formation of a heptacoordinated Zn(II)-centre in **L.Zn** in presence of ATP. Examples of heptacoordinated Zn(II)-centres are available in the literature.[9] A very insignificant shift in ^{31}P signals was observed when similar experiments were repeated for CTP.† The ^{31}P NMR spectral data also confirm the observed trend in binding affinity (ATP ≫ CTP > ADP ≫ AMP).† Presumably, the formation of a higher number of chelate rings between ATP or CTP and **L.Zn** is crucial for efficient **L.Zn**–O[phosphate] binding, as compared to those for ADP and AMP. Lack of any binding of PPi to **L.Zn** perhaps could be better explained based on the much higher solvation energy for PPi than that for ATP.[10] The 1 : 1 complex formation, *i.e.*, **L.Zn-ATP** was further confirmed by ESI-MS spectral studies.† Signals at 1103.9 and 1082 correspond to *m*/*z* for [[**L.Zn-ATP**] − 2(NO_3^-)] (A) and [(A–Na^+) + H^+],† respectively with anticipated isotope distribution.

Reversible binding of **L.Zn** to ATP could be demonstrated by adding an aqueous solution of sodium citrate (5.0×10^{-4} M) to **L.Zn-ATP**. The original spectrum of **L.Zn** (with λ_{max} = 463 nm) was restored when excess aqueous solution of sodium citrate was added to the solution of **L.Zn-ATP**.†

More interestingly, the solubility of the **L.Zn** (0.045 g L^{-1}) in water was found to be substantially enhanced (0.34 g L^{-1}) in presence of excess α-CD (4.5 g L^{-1}). Favoured non-bonded interactions between the azo functionality of **L.Zn** and the hydrophobic interior of the α-CD on inclusion into the α-CD cavity could account for the observed enhanced solubility of **L.Zn** in water (Fig. 3);† such inclusion of analogous (*E*)-1,2-di(pyridin-4-yl)ethene and (*E*)-1,2-di(pyridin-4-yl)diazene in α-CD was reported earlier.[11]

Spectral studies reveal that the absorption maximum for **L.Zn** (463 nm) shifts to shorter wavelength (458 nm) on addition of aqueous solution of α-CD.† Such inclusion in the α-CD cavity is expected to influence the HOMO–LUMO energy gap and thereby the absorption spectral bands—though the perturbation of the energy levels is not expected to be an appreciable one.[11,12] Thus, in this present study, the observed shift of 5 nm could be attributed to the inclusion phenomena and the formation of a [2]pseudorotaxane complex, **α-CD.L.Zn**. Presumably, formation of the more polar excited state for **α-CD.L.Zn** is less favored in the apolar interior of α-CD than in the surrounding solvent for a non-included reagent **L.Zn**.[12] Although small, this may have some contribution in increasing the energy of activation for optical electron transfer and thus the observed blue shift. Spectrophotometric titration with varying [α-CD] revealed a systematic shift in the spectral pattern with the appearance of two simultaneous isosbestic points at 420 and 446 nm.† The association constant (255 ± 15 M^{-1}, 25 °C) for this inclusion process was evaluated from the spectrophotometric titration data.† This value is close to those reported earlier for related systems.[11,12] Thus, in presence of excess of α-CD (10 fold), the [2]pseudorotaxane form, *i.e.*, **α-CD.L.Zn** (Fig. 3) is expected to be the major component (≥ 96%) in the aqueous solution. Higher solubility of **L.Zn** in water in presence of α-CD helped to achieve higher concentration of **L.Zn** (0.5 mM) in solution and thus a more intense colour change on binding to ATP (5 mM) (Fig. 3). Formation of the [2]pseudorotaxane was also confirmed by ESI-MS studies; a molecular ion peak for **α-CD.L.Zn** was observed at an *m*/*z* value of 1648.6.†

Preferential binding to ATP and the visual change in colour for **L.Zn** or **α-CD.L.Zn** on binding to ATP led us to consider the possible use of these reagents for staining of the live yeast cells, as maximum concentration of negatively charged ATP is released through mitochondria in the periplasmic space and

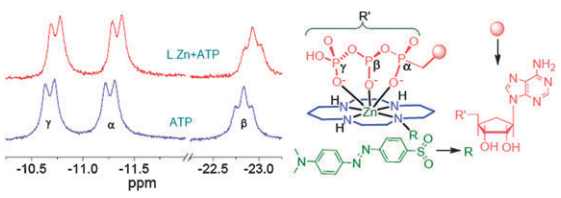

Fig. 2 ^{31}P NMR spectra recorded in D_2O; only (A) ATP and (B) ATP with Zn(II)-complex **L.Zn.ATP**, at 25 °C.

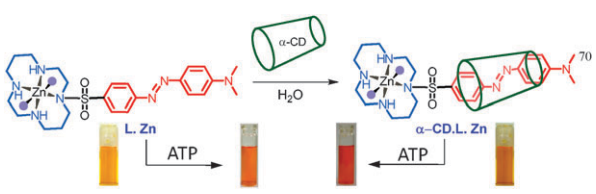

Fig. 3 Schematic representation of the inclusion process of **L.Zn** with α-CD and changes in solution colour for **L.Zn** and **α-CD.L.Zn** in presence of ATP.

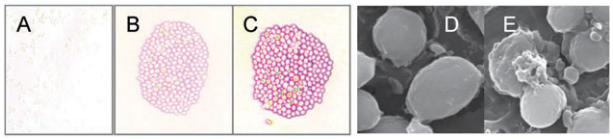

Fig. 4 Images of *S. cerevisiae* cells: Light microscopic images of (A) unstained cells, (B) cells stained with **L.Zn** (0.66×10^{-4} M) and (C) **α-CD.L.Zn** [**L.Zn** (5.0×10^{-4} M) and α-CD (5.0×10^{-3} M)] at 25 °C in 10 mM HEPES buffer solution (pH = 7.2); SEM images of (D) yeast cells, (E) yeast cells treated with **L.Zn** (0.66×10^{-4} M).

then gets excreted to the cell surface.[13] We have used the yeast cells, *Saccharomyces cerevisiae* (*S. cerevisiae*) in 10 mM HEPES buffer solution (pH = 7.2) for our studies. The images of the colorless yeast cells, viewed under a normal light microscope (AXIO IMAGER-Carl Zeiss), are shown in Fig. 4A; while images of the yeast cells exposed to the reagents **L.Zn** (for ~5 min) and **α-CD.L.Zn** (for ~2 min) are shown in Fig. 4B and C. Control experiments with yeast cells exposed separately to $Zn(NO_3)_2$ and **L** did not produce any change in colour. These observations together revealed that *S. cerevisiae* cells could be stained with **L.Zn** or **α-CD.L.Zn**, and the colour of the cells changed to pink-red. Further, for **α-CD.L.Zn**, the higher solubility allows a higher local concentration of the **L.Zn** and thus a more effective binding to the ATP, produced *in situ* in the cell surface and thereby better staining of the yeast cells.

Further yeast cells, stained with **L.Zn**, became colorless when treated with citrate ion (5.0×10^{-4} M) in 10 mM HEPES buffer solution. This confirms the reversible *in vivo* binding of ATP to the Zn(II)-centre of **L.Zn**. SEM images of blank and stained *eukaryote* (yeast) cells have been recorded (Fig. 4D and E) to reveal the change(s) in the morphology of the outer surface of the cells on binding to **L.Zn**. SEM images of yeast cells without the dye were found to be smooth in contrast to the images of the yeast cells with dye, where stained cell surfaces were found to be rough. This agrees well with the literature report that negatively charged ATP is concentrated on the cell surfaces.[14] This revealed the presence of the **L.Zn**, on the cell surface.

Finally, the cell growth, cell division, as well as the viability of the yeast cells in presence of these two staining agents were observed when the stained cell suspension was viewed on a concavity slide by light microscope (Fig. 5). Cell division and growth in presence of **L.Zn** and **α-CD.L.Zn** confirms the possibility of using these reagents as viable staining agents for studies on cell growth dynamics, which has significance in terms of the application potential in environmental studies and food industries.

In conclusion, we have successfully demonstrated that an easily synthesizable Zn(II)-reagent could be used as a viable staining agent for living cells in physiological conditions. Further, the use of biologically benign α-CD enhances the solubility in water and HEPES buffer (pH = 7.2) and thereby the staining efficiency. The stained microbial cell could be seen distinctly through a light microscope. Examples of such a non-toxic colorimetric staining agent that works completely in physiological conditions are extremely rare in the literature.

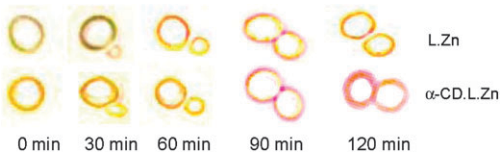

Fig. 5 Light microscopic image of *S. cerevisiae* in presence of **L.Zn** and **α-CD.L.Zn** in 10 mM HEPES buffer at different time intervals.

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Notes and references

‡ Patent application details: 0093NF2009 [1242DEL2010], 24.05.2010.

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