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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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1. General synthetic Procedure

1.1 Materials and Methods:

The chemicals such as 1,4,8,11-tetraazacyclotetradecane, (E)-4-((4-(dimethyl amino)phenyl)diazenyl)benzene-1-sulfonyl chloride, α-cyclodextrin, tetrabutylammonium salts of anions, AMP, ADP, ATP, CTP and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid were received from Aldrich and used as such. All the other reagents used were procured from S. D. fine chemicals, India. Solvents such as THF, methanol, chloroform (AR; Merck, India), ethanol (Spectrosol; Spectrochem, India) were used as solvents. HPLC grade water (Merck, India) was used for experiments and spectral studies. Microanalysis(C, H, N) was performed using a Perkin-Elmer 4100 elemental analyzer. FT—IR spectra were recorded in KBr pellets using Perkin Elmer Spectra GX 2000 spectrometer. ^{1}H and ^{31}PNMR spectra were recorded on Bruker 500 MHz FT NMR (model: Advance-DPX 200). Electronic spectra were recorded with Varian Cary 500 Scan Uv-vis-NIR Spectrophotometer. ESI-MS measurements were carried out on Waters QTof-Micro instrument. The images of the yeast cells, viewed under normal light microscope (AXIO IMAGER–Carl Zeiss).

1.2 Synthesis of compound L:

1,4,8,11-tetraazacyclotetradecane (cyclam) (247 mg, 1.23 mmole) was dissolved in dry tetra hydrofuran (40 ml) in a 250 ml Round Bottomed Flask. A solution (E)-4-((4-(dimethyl amino)phenyl)diazenyl)benzene-1-sulfonyl chloride (400 mg, 1.23 mmole) and triethyl amine (Et_3N) (1 ml) in tetrahydrofuran (10-15 ml) was added to the above solution in ice cold condition. Then, the resulting mixture was allowed to stir at room temp (25°C to 30°C) for 10 hr and then it was further refluxed for 1 hr. The orange precipitate thus obtained was filtered and washed with tetrahydrofuran. Finally the solid residue (**L**) was dried; Yield: 75%. ^{1}H NMR (500 MHz, $CDCl_3$, $SiMe_4$, *J* (Hz), δ (ppm)): 7.97 (2H, d, *J* = 8.0, Ar-H_e), 7.9 - 7.87

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(4H, m, Ar-$H_{c,d}$), 7.06 (3H, br, -$NH_{macrocycle}$), 6.76 (2H, d, J = 8.0, Ar-H_b), 3.15 (16H, br, $H_{f,h,i,j,k,m,n,o}$), 3.11 (6H, s, -$N(CH_3)_2$), 2.03 (4H, br , $H_{g,l}$). ESI-MS (+ ve mode): *m/z*; 488.30 (M^++H^+). Elemental Analysis data: Calc. C: 59.11, H: 7.65, N: 20.11, Expt. C: 59.1, H: 7.62, N: 20.2.

1.3 Synthesis of compound L.Zn :

A solution of **L** (200 mg; 0.41 mmole) was dissolved in 25 ml methanol in a 100 ml single necked flask. To this 148.69 mg of zinc nitrate dissolved in 1.0 ml water (1.2 mole equivalent) was added dropwise. The resultant solution was stirred at room temperature (25°C to 30°C) for 24 h. The reaction mixture was kept in refrigerator at 4°C for 5 h and a violet colored precipitate appeared. This was collected through filtration using Gooch (no. 3) crucible and was washed several times with $CHCl_3$; Yield: 65%.^{1}HNMR (500 MHz, DMF-d_7, $SiMe_4$, δ (ppm)): 7.92 (2H, d, J = 8.5, Ar-H_e), 7.87 (2H, d, J = 9, Ar-H_c), 7.80 (2H, d, J = 8.5, Ar-H_d), 6.91 (2H, d, J = 9.5, Ar-H_b), 3.13 (6H, s, -$N(CH_3)_2$), 2.92 (8H, br, $H_{f,g,n,o}$), 2.75 (12H, br, $H_{h,i,j,k,l,m}$). ESI-MS (+ ve mode), *m/z*: 676.00 (M^+). Elemental Analysis: Calc. C: 42.57, H: 5.51, N: 18.62; Expt. C: 42.88, H: 5.56, N: 18.52.

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2. Biological sample preparation

2.1 *Saccharomyces cerevisiae* cells

Yeast *(Saccharomyces cerevisiae*) was isolated from curd and then cultured in the Glucose yeast extract agar (GYE) medium (Glucose 1g, yeast extract 10g, Distilled water 1000 ml, pH 7.4). The cells were harvested and vortexed for making the homogenous suspension in sterile distilled water.

2.2 For SEM Images

The isolates of *Saccharomyces sp.* were cultured in GYE (glucose yeast extract agar) media, for 24 h at 37°C. 100 µl of this was taken and was allowed to incubate at 30 to 40°C along with 100 µl of **L.Zn** ($0.66x10^{-4}$ M) solution. The samples were fixed overnight at room temperature 25-30°C with 2% (v/v) glutaraldehyde in 0.1 M phosphate buffer at pH 7.5. The samples were then washed with 0.1 M phosphate buffer (pH 7.5) at room temperature 25-30°C for 1 h. Post-fixation was carried out in 2% (w/v) osmium tetroxide (OsO_4) in the same buffer and was washed once with 0.1 M phosphate buffer for 20 minutes. Then, the water was removed by a graded water-ethanol series; 25% ethanol-15 min, 50% ethanol-15min, 75% ethanol-30 min, 90% ethanol-60 min, absolute alcohol-30 min. The specimens were rinsed in buffer and coated with gold in a sputter coater (Polaron SC7620) prior to microscopy. The material was examined in a Scanning Electron Microscope (SEM) LEO 1430 VP at an accelerating Voltage of 15 kV.

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3. Solubility Experiment:

Enhancing the solubility of L.Zn in aqueous solution:

Solubility of **L.Zn** in water is limited (0.045 gL^{-1}). However, this could be enhanced in presence of α-cyclodextrin (α-CD). 2.3 mg of **L.Zn** was dissolved in 5 ml of double distilled water and stirred at room temperature for 1 hr. Then the solution was filtered and the concentration of the dissolved solid in the filtrate was evaluated based on the equation $O.D_{filtrate}$ / $\varepsilon_{\mathbf{LZn}}$ ($O.D_{filtrate}$ is optical density of the filtrate and $\varepsilon_{\mathbf{LZn}}$ is the molar extinction coefficient of **L.Zn**) (value was evaluated earlier through an independent experiment) and was found to be 0.045 gL^{-1}. Similar experiment was repeated in presence of α-CD. In this case 24.3 mg of α-CD was dissolved in 5 ml of double distilled water containing 2.913 mg of **L.Zn** and this solution was stirred at 25°C for 1 hr; while a solution with little suspended solid was obtained. This was filtered using G-4 gooch crucible. Filtrate was collected and optical density (O.D.) of the filtrate was monitored at 458 nm. Considering the complete formation of the inclusion complex (pseudorotaxane form), concentration was evaluated based on the known $\varepsilon_{\mathbf{LZn.\alpha\text{-}CD}}$ value and the O.D. at 458 nm. Calculation revealed that 0.34 gL^{-1} **L.Zn** is present in the resultant solution. Hence ~7.5 fold increase in solubility in terms of **L.Zn** was achieved and this is also evident in the difference of the visible colour of the saturated solution of **L.Zn** in absence and presence of α-CD in aqueous solution (ESI Figure 8). **L.Zn** present in aqueous solution in the [2]pseudorotaxane form is being represented as **α.CD.L.Zn** in Figure 3.

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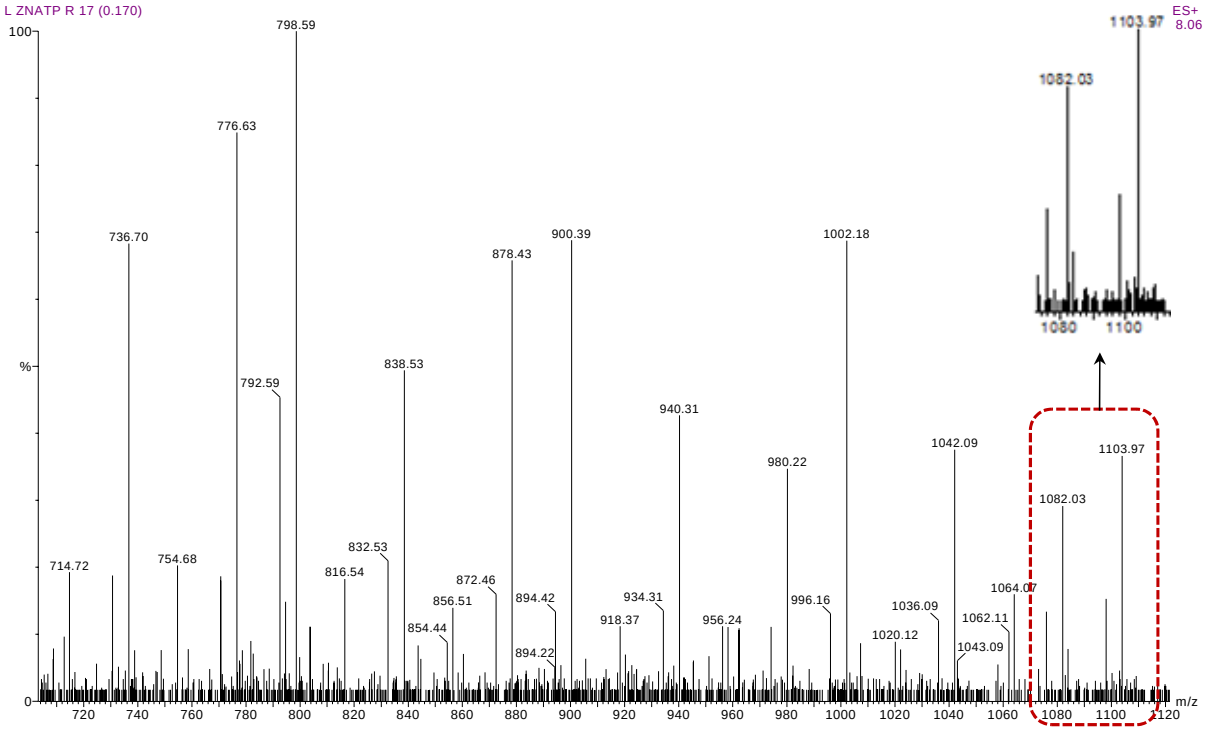
4. ESI-MS spectra:

4.1 ESI-MS spectra of L.Zn.ATP complex:



ESI Figure 1: ESI-MS spectra of **L.Zn** (7.2 μM) in presence of ATP (900 μM) in double distilled and deionised water. The highlighted peak 1082.03 supports the formation of a 1:1 complex between **L.Zn** and ATP.

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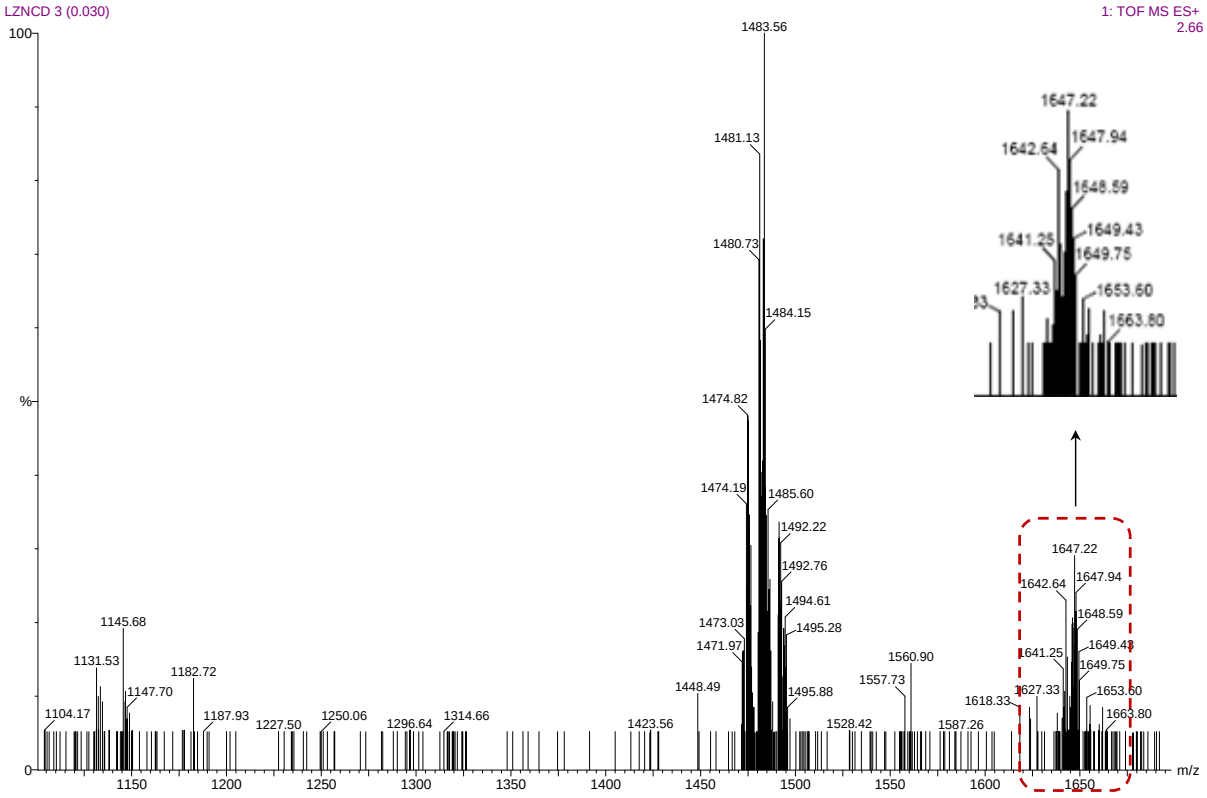
4.2 ESI-MS spectra of α-CD.L.Zn complex:



ESI Figure 2: ESI-MS spectra of **L.Zn** (8.6 μM) in presence of α-CD (100 μM) in double distilled and deionised water. The highlighted peak 1648.59 supports the formation of a 1:1 inclusion complex between **L.Zn** and α-CD.

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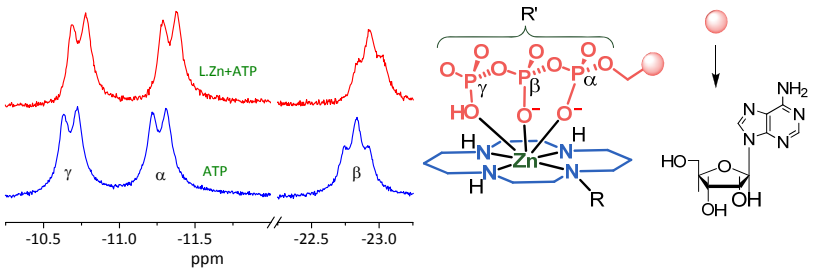
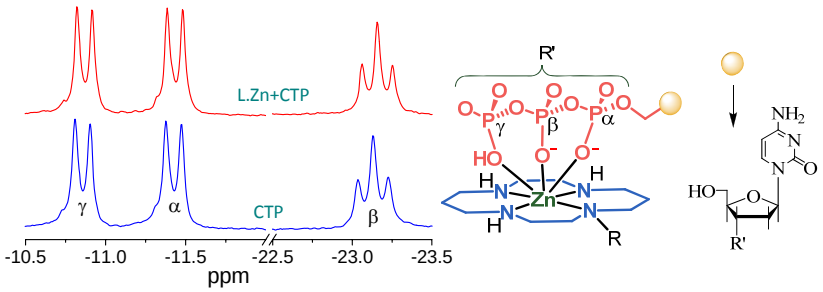
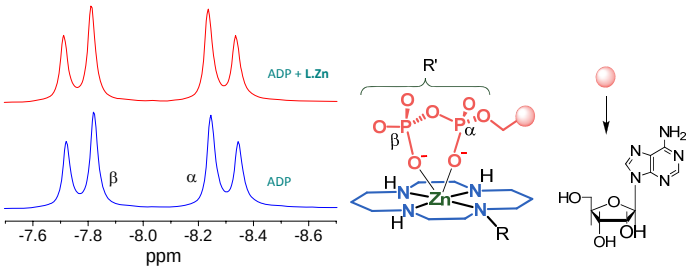
5. Changes in ^{31}PNMR specra of ATP, CTP and ADP in presence of L.Zn:



ESI Figure 3: Partial ^{31}PNMR spectra of ATP (0.066 mM) before and after addition of **L.Zn** (1 equivalent).



ESI Figure 4: Partial ^{31}PNMR spectra of CTP (0.067 mM) before and after addition of **L.Zn** (1 equivalent).



ESI Figure 5: Partial ^{31}PNMR spectra of ADP (0.067 mM) before and after addition of **L.Zn** (1 equivalent).

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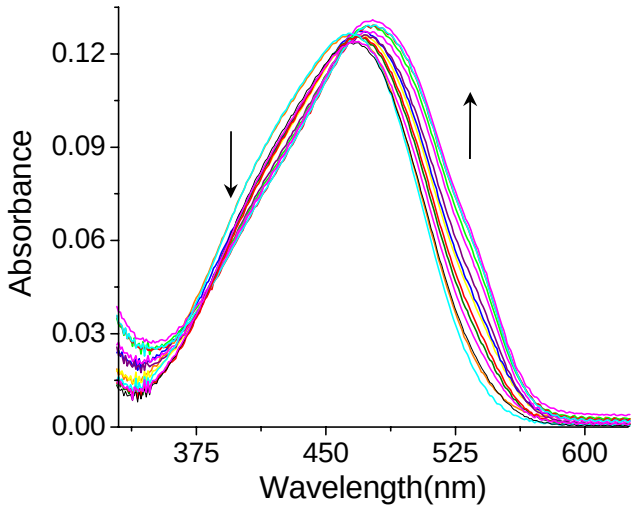
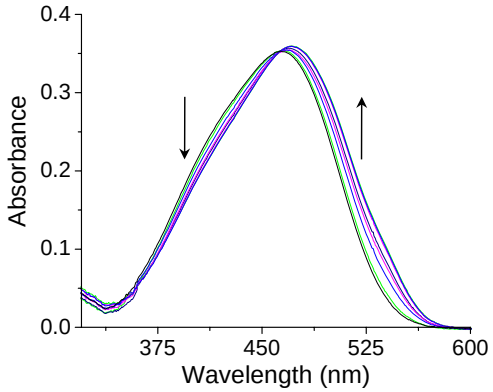
6. Spectrophotometric titration:

6.1 Spectrophotometric titration of L.Zn with CTP:



ESI Figure 6: Absorption spectra of **L.Zn** (1.79 μM) with varying [CTP] (0–10.17 mM) in aq. HEPES buffer (10 mM, pH 7.2) at 25°C.

6.2 Spectrophotometric titration of L.Zn with ADP:



ESI Figure 7: Absorption spectra of **L.Zn** (8.86 μM) with varying [ADP] (0–6.55 mM) in aq. HEPES buffer (10 mM, pH 7.2) at 25°C.

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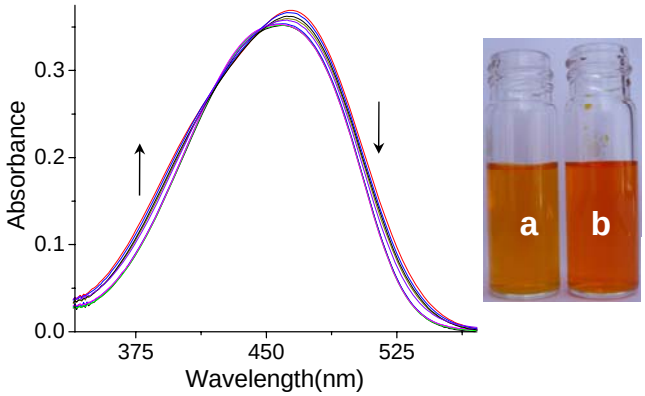
6.2 Spectrophotometric titration of L.Zn with α-CD:



ESI Figure 8: Absorption spectra of **L.Zn** (8.3 μM) with varying [α-CD] (0-2.3mM) in aq.HEPES buffer (10 mM, pH 7.2) at 25°C. Inset is the photograph of the saturated solution of (a) **L.Zn (**0.045 gl^{-1}) and (b) **L.Zn** (0.34 gl^{-1}) in presence of α-CD (4.5 gL-1) in double distilled and deionised water.

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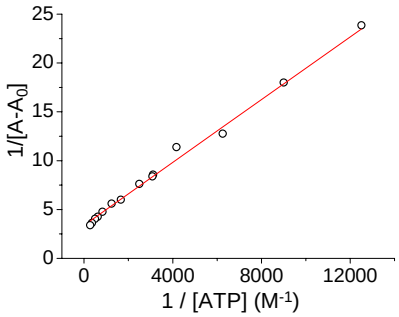
7. Benesi-Hildebrand plot:



SI Figure 9: Benesi-Hildebrand plot of 1 / ($A-A_0$) *vs.* 1 / [ATP] for change in absorption intensity at 503 nm upon addition of varying [ATP]. Goodness of the straight line fit (R^2 = 0.993) confirms 1:1 binding stoichiometry. Binding affinity, thus evaluated, was found to be $1.9 \pm 0.15).10^3$ M^{-1}.

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8. Interference Study :

SI Figure 10: Plot of absorption intensity of **L.Zn** (2 μM) with addition of ATP (4 mM) at 503 nm in presence of 5 equivalent excess of other anions in aq. HEPES buffer (0.01 M, pH 7.2). [A: ATP only, B: ATP + CTP, C: ATP + ADP, D: ATP + AMP, E: ATP + $H_2PO_4^-$, F: ATP + F^-, G: ATP + Cl^-, H: ATP + Br^-, I: ATP + I^-, J: ATP + Ac^-, K: ATP + SO_4^{2-}, L: ATP + CN^-, M: ATP + SCN^-, N: ATP + NO_3^-].

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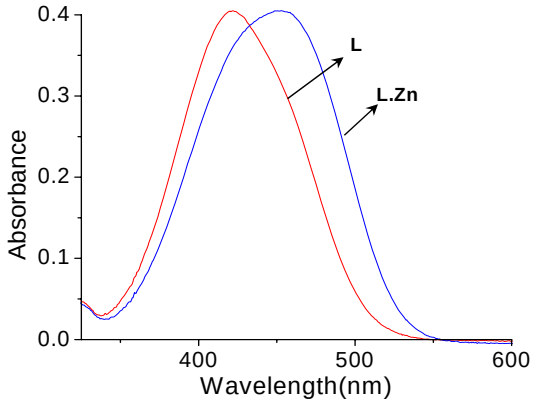
9. Absorption spectra of L and L.Zn:



ESI Figure 11: Absorption spectra of **L** (16 μM) and **L.Zn** (16 μM) in CH_3CN:MeOH (7:3, v/v)

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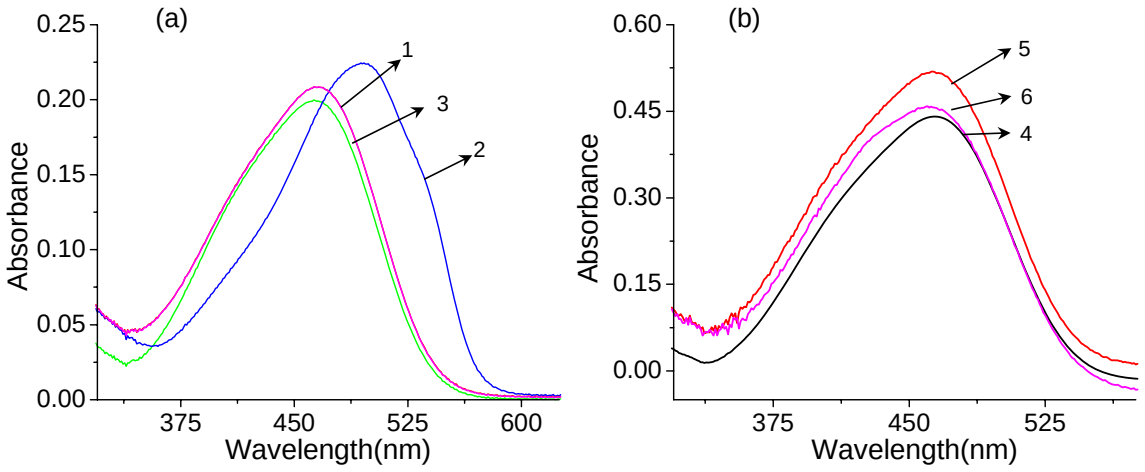
10. Absorption spectral evidence for the reversible binding of ATP to L.Zn:



ESI Figure 12: (a) Absorption spectra of (1) **L.Zn** (2.97 μM), (2) **L.Zn** in presence of ATP (5.0×10^{-4} M), (3) **L.Zn** in presence of ATP (3.5×10^{-4} M) and sodium citrate solution (5.0×10^{-4} M) in 10 mM HEPES buffer (pH = 7.2); (b) Absorption spectra of (4) **L.Zn** (7.1 μM), (5) **L.Zn** (7.1 μM) in presence of yeast (6) **L.Zn** (7.1 μM) + in presence of yeast cells and to this sodium citrate solution (5.0×10^{-4} M) was added 10 mM HEPES buffer (pH = 7.2). Spectra were recorded at 25°C.

Yeast cells were cultured in the Glucose yeast extract agar (GYE) medium (Glucose 1g, yeast extract 10g, Distilled water 1000 ml, pH 7.4). The cells were harvested and vortexed for making the homogenous suspension in sterile distilled water. Solution used for the spectroscopic measurements with Yeast cells was not as homogeneous as it was the case for solution of HEPES buffer with ATP and L.Zn. Moreover, other media constituents that were used for the Yeast culture were also present in the solution for spectroscopic measurements with Yeast cells and this could also have influenced the spectral nature.

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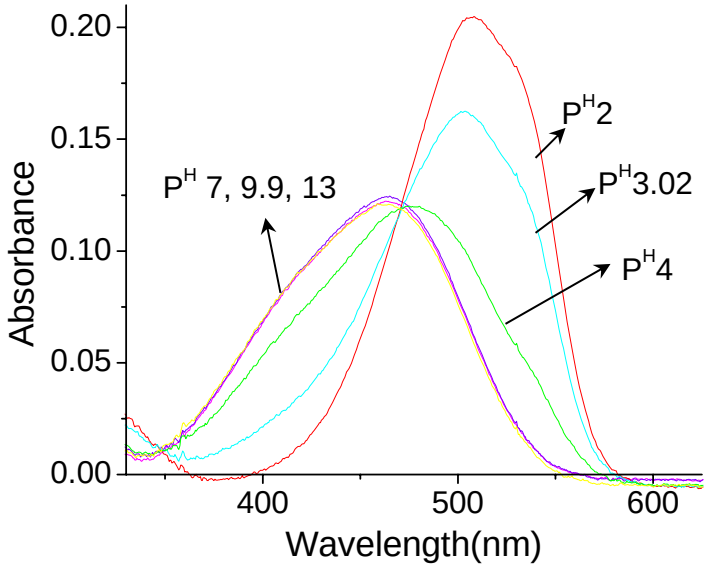
11. Absorption spectra of L.Zn at different pH:



ESI Figure 13: Absorption spectra of **L.Zn** (1.79 μM) at different pH.

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12. Methodology used for calculating binding constant:

12.1 Association constant values for ATP, CTP and ADP towards L.Zn:

ESI Table 1. Association constant values calculated from absorption titration method in aqueous HEPES buffer (pH 7.2).

Anion	ATP	CTP	ADP
K^a (M^{-1})	978 ± 15	220±15	142±15

Methodology Used for evaluation of these Association Constants:

General formula used for calculating the binding constants from UV-vis titrations.

$$\mathbf{L.Zn} + A^- = \mathbf{L.Zn.A^-}$$

Absorption maxima for **L.Zn** is $\lambda_{\mathbf{L.Zn}}$, while extinction coefficient and OD at this wavelength are $\varepsilon_{\mathbf{L.Zn}}$ and $A_{\mathbf{L.Zn}}$ respectively. Absorption maxima for $\mathbf{L.Zn.A^-}$ is $\lambda_{\mathbf{L.Zn.A^-}}$ while extinction coefficient and OD at this wavelength are $\varepsilon_{\mathbf{L.Zn.A^-}}$ and $A_{\mathbf{L.Zn.A^-}}$, respectively.

Thus, for a given concentration of the receptor molecule ([**L.Zn**]) and anion ([A^-])

$$[\mathbf{L.Zn.A^-}] = [\{A_{\mathbf{LZn.A^-}} - A^0\} / \varepsilon_{\mathbf{L.Zn.A^-}}] \quad (1)$$

Where, A^0 is OD value at $\lambda_{\mathbf{L.Zn.A^-}}$ before addition of externally added A^-.

$A_{\mathbf{L.Zn.A^-}}$ was recorded at $\lambda_{\mathbf{L.Zn.A^-}}$ after addition 100 mole equivalent of A^-.

Therefore, $\varepsilon_{\mathbf{L.Zn.A^-}} = A_{\mathbf{L.Zn.A^-}} / [\mathbf{L.Zn}]_{free}$ (2)

(Assuming all the receptor molecules is bound to A^- and A^- alone does not absorb at this wavelength)

Thus, once we one can evaluate [$\mathbf{L.Zn.A^-}$], it is possible to calculate concentration of the uncomplexed receptor molecule, [**L.Zn**] using equations 1 and 2.

$$[\mathbf{L.Zn}]_{free} = [\mathbf{L.Zn}]_{initial} - [\mathbf{L.Zn.A^-}] \quad (3)$$

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and, $[A^-]_{free} = [A^-]_{initial} - [\mathbf{L.Zn.A^-}]$ (4)

If K is the formation constant for the complex, $\mathbf{L.Zn.A^-}$ for a given concentration of A^-

Binding Constant, $K = [\mathbf{L.Zn.A^-}] / \{[\mathbf{L.Zn}]_{free}[A^-]_{free}\}$ (5)

12.2 Methodology used for calculating binding constant of α-cyclodextrin with L:

General formula that was used for calculating the binding constants from UV-vis titrations is given below:

L.Zn + α-CD = **α-CD.L.Zn**

Absorption maxima for **L.Zn** is $\lambda_{\mathbf{L.Zn}}$, while extinction coefficient and OD at this wavelength are $\varepsilon_{\mathbf{L.Zn}}$ and $A_{\mathbf{L.Zn}}$ respectively. Absorption maxima for **α-CD.L.Zn** is $\lambda_{\mathbf{\alpha\text{-}CD.L.Zn}}$, while extinction coefficient and OD at this wavelength are $\varepsilon_{\mathbf{\alpha\text{-}CD.L.Zn}}$ and $A_{\mathbf{\alpha\text{-}CD.L.Zn}}$ respectively.

Thus, for a given concentration of the receptor molecule ([**L**]) and α-cyclodextrin ([α-CD])

$[\mathbf{\alpha\text{-}CD.L.Zn}] = [\{\mathbf{\alpha\text{-}CD.L.Zn} - A^0\} / \varepsilon_{\mathbf{\alpha\text{-}CD.L.Zn}}]$ (6)

Where, A^0 is OD value at $\lambda_{\mathbf{\alpha\text{-}CD.L.Zn}}$ before addition of externally added α-CD.

$A_{\mathbf{\alpha\text{-}CD.L.Zn}}$ was recorded at $\lambda_{\mathbf{\alpha\text{-}CD.L.Zn}}$ after addition 100 mole equivalent of α-CD.

Therefore, $\varepsilon_{\mathbf{\alpha\text{-}CD.L.Zn}} = A_{\mathbf{\alpha\text{-}CD.L.Zn}} / [\mathbf{L.Zn}]_{free}$ (7)

(Assuming all the receptor molecules is bound to α-CD and α-CD do not have any absorption at this wavelength)

Thus, once we one can evaluate [**α-CD.L.Zn**], it is possible to calculate concentration of the uncomplexed receptor molecule, [**L.Zn**] using equation 6 and 7.

$[\mathbf{L.Zn}]_{free} = [\mathbf{L.Zn}]_{initial} - [\mathbf{\alpha\text{-}CD.L.Zn}]$ (8)

and, $[\alpha\text{-CD}]_{free} = [\alpha\text{-CD}]_{initial} - [\mathbf{\alpha\text{-}CD.L.Zn}]$ (9)

If K is formation constant for the inclusion complex, **α-CD.L.Zn** for a given concentration of α-CD

Binding Constant, $K = [\mathbf{\alpha\text{-}CD.L.Zn}] / \{[\mathbf{L.Zn}]_{free}[\alpha\text{-CD}]_{free}\}$ (10)

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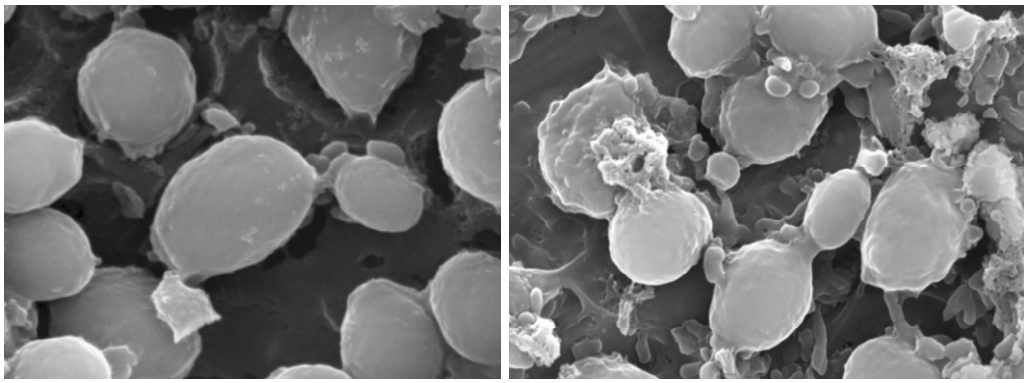
13. SEM images:



(A) (B)

ESI Figure 14: SEM images of (A) blank yeast cells, (B) yeast cells treated with **L.Zn** complex, [**L.Zn**] = 0.066 mM.