Supplementary information

Zn(II) and Cd(II) based complexes for probing the enzymatic hydrolysis of Na₄P₂O₇ by Alkaline phosphatase in physiological condition

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**Materials and method:**

The chemical such as 3-nitro-4-chloro coumarin, dipicolyl amine, Hg(ClO$_4$)$_2$, Cd(ClO$_4$)$_2$, Zn(ClO$_4$)$_2$, Ni(ClO$_4$)$_2$, Co(ClO$_4$)$_2$, Pb(ClO$_4$)$_2$, Fe(ClO$_4$)$_2$, Cu(ClO$_4$)$_2$, Cr(ClO$_4$)$_2$, and different nucleotides (adenosine 5'-monophosphate monohydrate, adenosine 5'-diphosphate sodium salt, adenosine 5'-triphosphate disodium hydrate, cytidine 5'-triphosphate disodium salt hydrate) were obtained from Sigma-Aldrich and were used as received without any further purification. Other salts like, NaF, NaI, NaBr, NaOAc, NaCl, NaH$_2$PO$_4$, Na$_4$P$_2$O$_7$, Na$_2$SO$_4$, NaNO$_3$ and all the other reagents used were of reagent grade (S. D. Fine chemical, India) and were used as received. Various analytical and spectroscopic data obtained for L, LZn and LCD agreed well with the proposed formulation and required purity. HPLC grade water (Fisher scientific) was used as a solvent. Ethyl acetate and methanol, which were used for different synthetic procedures, were purified through distillation following standard procedures, prior to use. Microanalysis (C, H, N) were performed using a Perkin-Elmer 4100 elemental analyzer. FTIR spectra were recorded as KBr pellets using Perkin Elmer Spectra GX 2000 spectrometer. $^1$H and $^{31}$P NMR spectra were recorded on Bruker 200 MHz (Avance-DPX 200)/ 500 MHz (Bruker Avance II 500) FT NMR. Electronic spectra were recorded with Cary-Varian UV-VIS NIR spectrophotometer. Fluorescence spectra were recorded using Fluorolog (Horiba Jobin Yvon) or Edinburgh F920 (Edinburgh Instrument) fluorescence spectrometer.

**Synthetic scheme:**

![Synthetic Scheme](image)

i: Ethyl acetate, 0°C 1h & RT 4h

ii: M(ClO$_4$)$_2$, Methanol, RT

M$^{n+}$: Zn$^{2+}$ or Cd$^{2+}$

**SI Figure 1:** Methodology adopted for synthesis of L, LZn, LCD.
Synthesis of L (4-(bis(pyridin-2-ylmethyl)amino)-3-nitro-2H-chromen-2-one):

Di-(2-picolyl) amine (398.5mg, 0.45 mL 2.5 mmol) was dissolved in 30 mL ethyl acetate. This solution was taken in a 100 mL two necked round bottom flask and cooled to 0°C. 3-nitro-4-chloro coumarin (564 mg, 2.5 mmol) dissolved in 20 mL of ethyl acetate was added to this solution in a dropwise manner. The reaction mixture was stirred at 0°C for 1 hr. The reaction mixture was further stirred for another 2 hrs to ensure completion. Subsequently a yellow coloured precipitate was appeared, which was filtered off, this residue was purified by column chromatography (neutral Al₂O₃, hexane-ethyl acetate as eluent to get the L as a pure product (551mg, 71%).

$^1$H NMR (500 MHz, CD₃CN, 25 °C, TMS) δ (ppm): 8.60 (d, J = 5Hz, 2H; ArH), 8.15 (d, J = 8 Hz, 1H; ArH), 8.08 (t, J = 7.5Hz, 2H; ArH), 7.739 (t, J = 7.5Hz 1H; ArH), 7.65 (d, J = 8Hz, 2H; ArH), 7.60-7.59 (m, 2H; ArH), 7.489-7.416 (m, 2H; ArH), 4.708 (s, 4H; Ar-CH₂), 13C NMR (500 MHz, CD₃CN, 25 °C, TMS) δ (ppm): 58.165 (s, Ar -CH₂), 153.23, 150.632, 149.48, 140.89, 140.83, 139.76, 135.72, 134.182, 130.06, 127.69, 126.71, 125.76, 125.05, 124.84. IR (KBr) ν_max/cm⁻¹: 3434, 3167, 2364, 1716, 1602, 1553, 1516, 1460, 1402, 1049, 988, 767, 627. ESI-MS (m/z): 388.31 ((M⁺), 100%) 411.38 ((M⁺ + Na⁺). Elemental analysis: C₂₁H₁₆N₄O₄: calculated C (64.94), H (4.15) N (14.43); found C (64.7), H (4.2) N (14.25).

Synthesis of L.Zn

L (102mg, 0.261mmol) was dissolved in 20 mL of methanol. To this, Zn(ClO₄)₂, xH₂O (146 mg, 0.391 mmol) solution in 5 mL HPLC water was added in a dropwise manner. The resultant solution mixture was allowed to stir for 4 h at room temperature. A white coloured precipitate appeared, which was filtered off and dried in air to get the desired complex, L.Zn in pure form (Yield: 85.5 mg, 51%). $^1$H NMR (200 MHz, dmso-d₆, 25 °C, TMS) δ (ppm): 8.69 (d, J = 4.8Hz, 2H; ArH), 8.18 (t, J = 8.2 Hz, 2H; ArH), 7.92 (d, J = 8.2Hz, 1H; ArH), 7.74 - 7.65 (m, 5H; ArH), 7.36 (t, J = 6.2Hz, 2H; ArH), 4.585 (s, 4H; Ar-CH₂). 13C NMR (500 MHz, dmso-d₆, 25 °C, TMS) δ (ppm): 57.867(s, Ar -CH₂), 157.38, 156.01, 153.68, 148.08, 143.38, 140.72, 137.38, 135.79, 130.54, 127.11, 126.92, 126.68, 119.49 IR (KBr) ν_max/cm⁻¹: 3442, 3049, 2685, 2361, 1517, 1547, 1477, 1471, 1385, 1093, 1009, 772, 621. ESI-MS (m/z): 527.81 (M⁺ + 2H₂O + K⁺, 50%), 674.76 (M⁺ + 2ClO₄⁻ + H₂O, 15%), 692.81 (M⁺ + 2ClO₄⁻ + 2H₂O, 20%). Elemental analysis: C₂₁H₂₀Cl₂N₄O₁₄Zn: Calculated C (36.62), H (2.93), N (8.13); found C (36.8), H (2.76), N (8.08).
**Synthesis of L.Cd**

L (100 mg, 0.257 mmol) was dissolved in 20 mL of methanol and then a solution of Cd(ClO₄)₂·xH₂O (161 mg, 0.385 mmol) in 5 mL HPLC water was added in a drop wise manner into it. The resultant solution was allowed to stir for 4h at room temperature. A white coloured precipitate was appeared, which was filtered off and dried in air to get the pure metal complex, Cd.L (Yield: 87.3mg, 46.2%). ¹H NMR (200 MHz, dmsô-d₆, 25°C, TMS) δ (ppm): 8.65 (d, J = 4.8Hz, 2H; ArH), 8.11 (t, J = 8.2Hz, 2H; ArH), 7.95 (d, J = 8.2Hz, 1H; ArH), 7.67 - 7.58 (m, 5H; ArH), 7.38 (t, J = 6.0Hz, 2H; ArH), 4.585 (s, 4H; Ar-CH₂). ¹³C NMR (500 MHz, dmsô-d₆, 25 °C, TMS) δ (ppm): 57.903(s, Ar -CH₂), 157.35, 155.82, 153.43, 147.38, 147.67, 147.57, 143.85, 135.73, 130.55, 127.10, 126.89, 119.44. IR (KBr) νmax/cm⁻¹: 3437, 3049, 2984, 2684, 2362, 1655, 1549, 1503, 1475, 1340, 1285, 1229, 1186, 1093, 909, 861, 772, 621. ESI-MS (m/z): 519.34 (M⁺ + H₂O, 41%), 537.35 (M⁺ + 2H₂O, 52%) 601.37 (M⁺ + 2ClO₄⁺ + 2H₂O, 20%). Elemental analysis: C₂₁H₂₀Cl₂N₄O₁₄Cd: calculated C (34.28), H (2.74) N (7.62); found C (34.13), H (2.7) N (7.57).

**UV-visible spectra of L**

[Graph: UV-visible spectra of L (2.12 × 10⁻⁵ M) in aqueous 0.01mM HEPES buffer medium of pH 7.4.

SI Figure 1: Absorption spectra of L (2.12 × 10⁻⁵ M) in aqueous 0.01mM HEPES buffer medium of pH 7.4.
**UV-visible spectra of L.Zn**

![Graph](image)

**SI Figure 2**: Absorption spectra of **L.Zn** (2.0 x 10^{-5} M) in aqueous 0.01mM HEPES buffer of pH 7.4, molar extinction coefficient 1.1129 x 10^{4} M^{-1}cm^{-1} at wavelength 326nm.

**UV-visible spectra of L.Cd**

![Graph](image)

**SI Figure 3**: Absorption spectra of **L.Cd** (2.0 x 10^{-5} M) in aqueous 0.01mM HEPES buffer of pH 7.4, molar extinction coefficient 1.278 x 10^{4} M^{-1}cm^{-1} at wavelength 326nm.
Luminescence response Of L.Zn towards different Anions.

**SI Figure 4**: Luminescence response of L.Zn (2.0 x 10^{-5} M) in aqueous 0.01mmol HEPES buffer (pH-7.4) medium on addition of the solution of sodium salt of various anions and nucleotides: 1. L.Zn, 2. NO_3^-, 3. ATP, 4. H_2PO_4^-, 5. I^-, 6. AMP, 7. SO_4^-, 8. PPi, 9. Cl^-, 10. Br^-, 11. CH_3CO_2^-, 12. ADP, 13. CTP. (2.0 x 10^{-4} M) in 0.01mmol HEPES buffer (pH-7.4) with λ_{mon} = 428 nm and λ_{ext} = 328 nm.

Luminescence response Of L.Cd towards different Anions.

**SI Figure 5**: Luminescence response of L.Cd (2.0 x 10^{-5} M) in aqueous 0.01mmol HEPES buffer (pH-7.4) medium on addition of the solution of sodium salt of various anions and nucleotides: 1. L.Cd, 2. NO_3^-, 3. ATP, 4. H_2PO_4^-, 5. I^-, 6. AMP, 7. SO_4^-, 8. PPi, 9. Cl^-, 10. Br^-, 11. CH_3CO_2^-, 12. ADP, 13. CTP. (2.0 x 10^{-4} M) with λ_{mon} = 414 nm, λ_{ext} = 328 nm.
**Benesi-Hildebrand plot for binding studies of NaPPI towards L.Zn:**

SI Figure 5: Benesi-Hildebrand plot of for evaluation of binding constant and stoichiometry for the formation of L.Zn-PPi complex in aqueous 0.01mmol HEPES buffer (pH-7.4) medium. $\lambda_{\text{ext}} = 328$ nm and $\lambda_{\text{mon}} = 428$ nm were used for emission studies. Goodness of the fit of the plot confirms the 1:1 binding stoichiometry.

**Benesi-Hildebrand plot of L.Cd with NaPPI**

SI Figure 6: Benesi-Hildebrand plot of for evaluation of binding constant and stoichiometry for the formation of L.Cd-PPi complex in aqueous 0.01mmol HEPES buffer (pH-7.4) medium. $\lambda_{\text{ext}} = 328$ nm and $\lambda_{\text{mon}} = 414$ nm were used for emission studies. Goodness of the fit of the plot confirms the 1:1 binding stoichiometry.
$^{31}$P NMR of PPI in absence and presence of L.Zn, L.Cd

**SI Figure 7:** $^{31}$P NMR spectra of L.Zn and L.Cd (15 mM) before and after addition of Na$_4$P$_2$O$_7$ (150 mM) in D$_2$O.

A Ribbon diagram plot of Relative change in emission intensity of L.Zn with varying [ALP].

**SI Figure 8:** A ribbon diagram plot of relative change in emission intensity of L.Zn with varying [ALP] in aqueous 0.01mmol HEPES buffer (pH-7.4) medium. For each measurement of emission intensity, a time interval of 900 sec was allowed. $\lambda_{\text{ext}} = 328$ nm and $\lambda_{\text{mon}} = 428$ nm were used for emission studies.
**A Ribbon diagram plot of Relative intensity of L.Cd with varying concentration of ALP**

**SI Figure 9:** A ribbon diagram plot of relative change in emission intensity of L.Cd with varying [ALP] in aqueous 0.01mmol HEPES buffer (pH-7.4) medium. For each measurement of emission intensity, a time interval of 900 sec was allowed. $\lambda_{\text{ext}} = 328$ nm and $\lambda_{\text{mon}} = 414$ nm were used for emission studies.

**Emission spectra of L.Zn in absence and presence of Alkaline phosphatase**

**SI Figure 10:** Emission spectra of L.Zn (2.0 X10$^{-5}$) in absence and presence of 100 nM ALP in aqueous 0.01mmol HEPES buffer (pH-7.4) medium.
SI Figure 11: Emission spectra of L.Cd (2.0 X 10^{-5}) in absence and presence of 100 nM ALP in aqueous 0.01mmol HEPES buffer (pH-7.4) medium.

ESI figure 10, 11, reveals that there is no interaction of Alkaline phosphatase (ALP) with L.Zn and L.Cd in absence of Na₄P₂O₇ (NaPPi) in aqueous 0.01mmol HEPES buffer (pH-7.4) medium. We had made two different types assay. First one has (i) L.M^{2+} (M = Zn²⁺, Cd²⁺) 2.0 x10^{-5}M; while the second one has (ii) L.M^{2+} (M = Zn²⁺, Cd²⁺) 2.0 x10^{-5}M + ALP (100 nM) in 0.01M aqueous HEPES buffer (pH-7.4) medium. The emission spectra were recorded for the first aliquot then the emission intensity of the second aliquot was measured after 900 sec of mixing ALP. No change in the emission spectral pattern was observed after addition of ALP to both the metal complexes (L.Zn and L.Cd) in absence of Na₄P₂O₇ (NaPPi), which confirms that there is no interaction between L.Zn or L.Cd and ALP. Again the significant change in the emission intensity of L.Zn and L.Cd in presence both Na₄P₂O₇ and ALP implied that enzymatic hydrolysis of PPi by ALP reduces the effective concentration of PPi in the medium and thus effective concentration of L.Zn-PPi or L.Cd-PPi in the medium and resulted overall changes in the emission intensity.
Interference Study for the binding of NaPPi with L.Zn in aqueous 0.01mmol HEPES buffer (pH-7.4) medium:

**SI Figure 12:** Emission intensity at 428 nm of L.Zn (2.0 x 10^{-5} M) with Na_4P_2O_7 (1.0 x 10^{-4} M) in the absence and presence of 5.0 x 10^{-3} M of different coexisting phosphate anions and nucleotides in in aqueous 0.01mmol HEPES buffer (pH-7.4) medium (λ_{ext} = 328 nm).

Interference Study for the binding of NaPPi with L.Cd

**SI Figure 13:** Emission intensity at 414 nm of L.Cd (2.0 x 10^{-5} M) with Na_4P_2O_7 (1.0 x 10^{-4} M) in the absence and presence of 5.0 x 10^{-3} M of different coexisting phosphate anions and nucleotides in in aqueous 0.01mmol HEPES buffer (pH-7.4) medium (λ_{ext} = 328 nm).
Mass spectra of L\(_{\text{Cd}}\) complex in presence of NaPPi

SI Figure 14: ESI-MS spectra of L\(_{\text{Cd}}\) in presence of 10 mole equivalent of NaPPi in aqueous medium.
Mass spectra of L.Zn complex in presence of NaPPi

SI Figure 15: ESI-MS spectra of L.Zn in presence of 10 mole equivalent of NaPPi in aqueous medium.
SI Figure 16: ESI-MS spectra of L.Zn in presence of 10 mole equivalent of NaPPi in aqueous medium.

Relative Quantum yield (Φ) values for L.Zn L.Cd with respect to quinine sulphate as standard.

[Φ]_{L.Zn} = 0.01559 (with respect to quinine sulphate ([Φ]_{Std} = 0.54 in 0.1(M) H_2SO_4))

[Φ]_{L.Cd} = 0.003274 (with respect to quinine sulphate ([Φ]_{Std} = 0.54 in 0.1(M) H_2SO_4)).