Supplementary information

Zn(II) and Cd(II) based complexes for probing the enzymatic hydrolysis of $Na_4P_2O_7$ by Alkaline phosphatase in physiological condition

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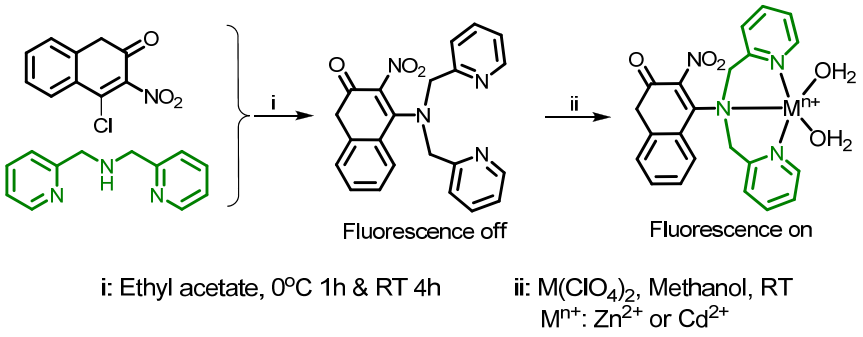
Table of Contents:-

Materials and method:

The chemical such as 3-nitro-4-choloro coumarin, di picolyl 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_2PO_4, $Na_4P_2O_7$, Na_2SO_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 scheame:



SI Figure 1: Methodology adopted for synthesis of **L**, **L.Zn**, **L.Cd**.

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-choloro 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 Al_2O_3, hexane-ethyl acetate as eluent to get the **L** as a pure product (551mg, 71%). ^{1}H NMR (500 MHz, CD_3CN, 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-CH_2), ^{13}C NMR (500 MHz, CD_3CN, 25 °C, TMS) δ (ppm): 58.165 (s, Ar -CH_2), 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, 1280, 1049, 988, 767, 627. ESI-MS (m/z): 388.31 ((M^+), 100%) 411.38 ($(M^+ + Na^+)$. Elemental analysis: $C_{21}H_{16}N_4O_4$: calculated C (64.94), H (4.15) N (14.43); found C (64.7), H (4.2) N (14.25).

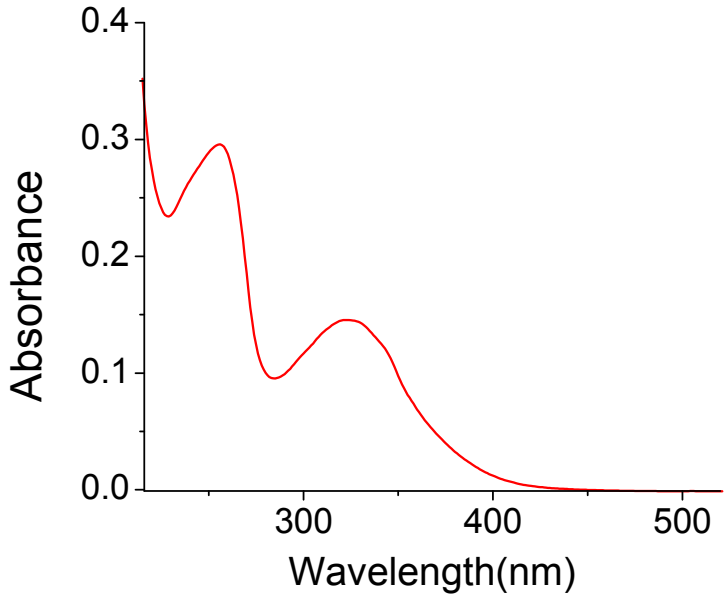
Synthesis of L.Zn

L (102mg, 0.261mmol) was dissolved in 20 mL of methanol. To this, $Zn(ClO_4)_2$, xH_2O (146 mg, 0.391 mmol) solution in 5 mL HPLC water was added in a drop wise 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, **LZn** in pure form (Yield: 85.5 mg, 51%). ^{1}H NMR (200 MHz, dmso-d_6, 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-CH_2). ^{13}C NMR (500 MHz, dmso-d_6, 25 °C, TMS) δ (ppm): 57.867(s, Ar -CH_2), 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, 1656, 1549, 1503, 1457, 1340, 1285, 1229, 1093, 909, 772, 621. ESI-MS (m/z): 527.81 ($M^+ + 2H_2O + K^+$, 50%), 674.76 ($M^+ + 2ClO_4^+ + H_2O$, 15%), 692.81 ($M^+ + 2ClO_4^+ + 2H_2O$, 20%). Elemental analysis: $C_{21}H_{20}Cl_2N_4O_{14}Zn$: Calculated C (36.62), H (2.93), N (8.13); found C (36.8), H (2.76), N (8.08).

Synthesis of L.Cd

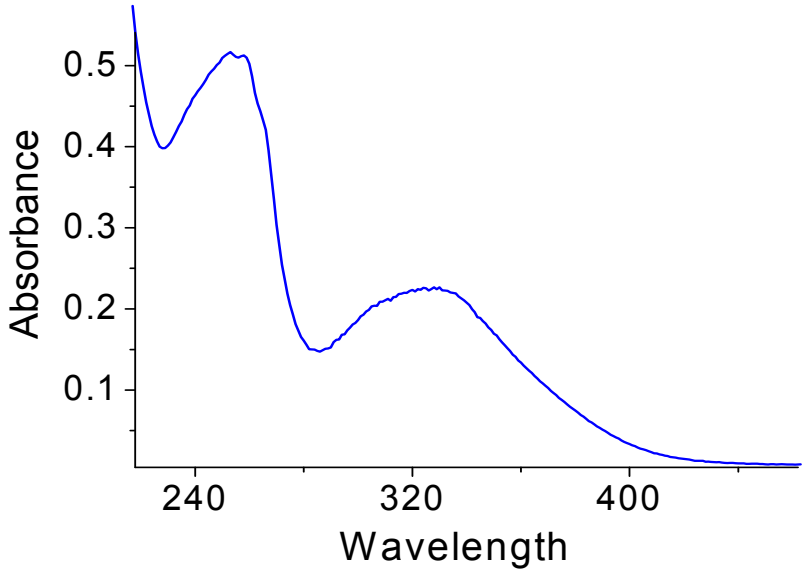
L (100 mg, 0.257 mmol) was dissolved in 20 mL of methanol and then a solution of $Cd(ClO_4)_2.xH_2O$ (161 mg, 0.385 mmol) in 5 mL HPLC water was added in a drop wise manner into it. The resultant solution was allowed to stir for 4h at room temperature. A white coloured precipitate was appeared, which was filtered off and dried in air to get the pure metal complex, **Cd.L** (Yield: 87.3mg, 46.2%). ^{1}H NMR (200 MHz, dmso-d_6, 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-CH_2). ^{13}C NMR (500 MHz, dmso-d_6, 25 °C, TMS) δ (ppm): 57.903(s, Ar -CH_2), 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) ν_{max}/cm^{-1}: 3437, 3049, 2984, 2684, 2362, 1655, 1549, 1503, 1457, 1340, 1285, 1229, 1186, 1093, 909, 861, 772, 621. ESI-MS (m/z): 519.34 ($M^+ + H_2O$, 41%), 537.35 ($M^+ + 2H_2O$, 52%) 601.37 ($M^+ + 2ClO_4^+ + 2H_2O$, 20%). Elemental analysis: $C_{21}H_{20}Cl_2N_4O_{14}Cd$: 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



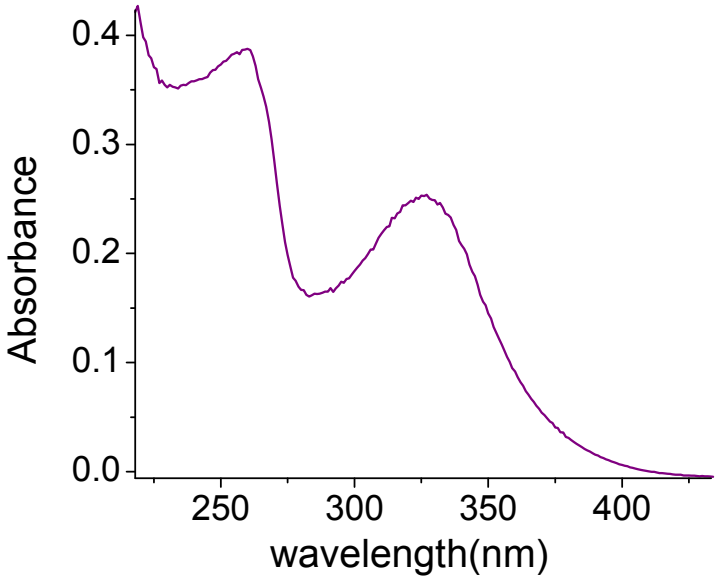
SI Figure 1: Absorption spectra of **L** (2.12 x10^{-5} M) in aqueous 0.01mM HEPES buffer medium of pH 7.4.

UV- visible spectra of L.Zn



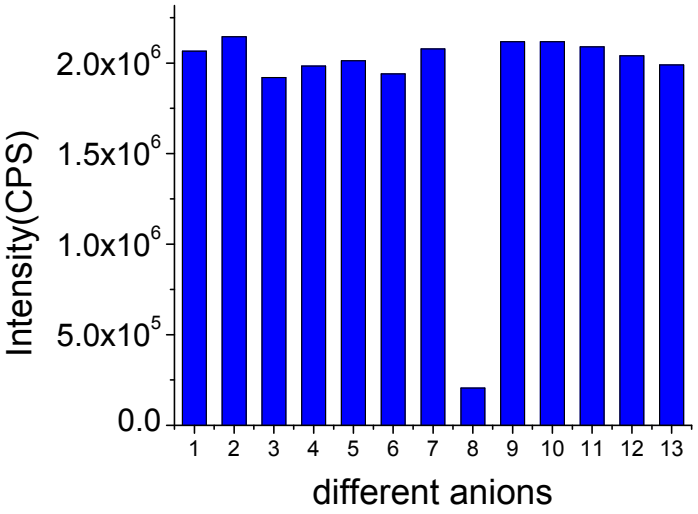
SI Figure 2: Absorption spectra of **L.Zn** (2.0 x10^{-5} M) in aqueous 0.01mM HEPES buffer of pH 7.4, molar extinction coefficient 1.1129 x 10^4 $M^{-1}cm^{-1}$ at wavelength 326nm

UV- visible spectra of LCd



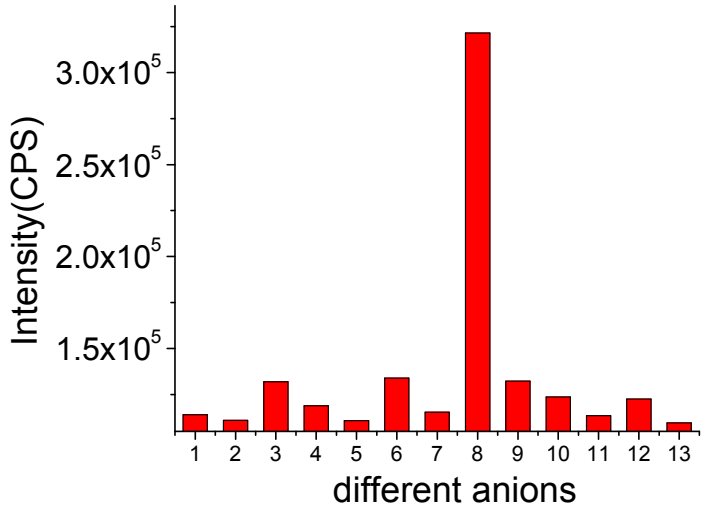
SI Figure 3: Absorption spectra of **L.Cd** (2.0 x10^{-5} M) in aqueous 0.01mM HEPES buffer of pH 7.4, molar extinction coefficient 1.278 x 10^4 $M^{-1}cm^{-1}$ at wavelength 326nm.

Luminescence response Of LZn towards different Anions.



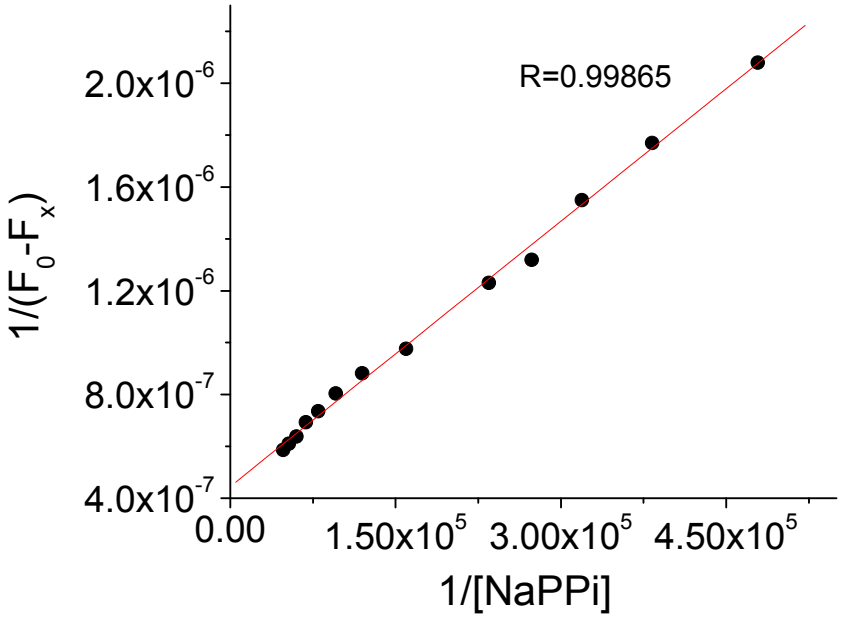
SI Figure 4: Luminescence response of **L.Zn** (2.0 x10^{-5} M) in aqueous 0.01mmol HEPES buffer (pH-7.4) medium on addition of the solution of sodium salt of various anions and nucleotides: 1. **L.Zn**, 2. NO_3^-, 3. ATP, 4. $H_2PO_4^-$, 5. I^-, 6. AMP, 7. SO_4^-, 8. PPi, 9. Cl^-, 10. Br^-, 11. $CH_3CO_2^-$, 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 LCd towards different Anions.



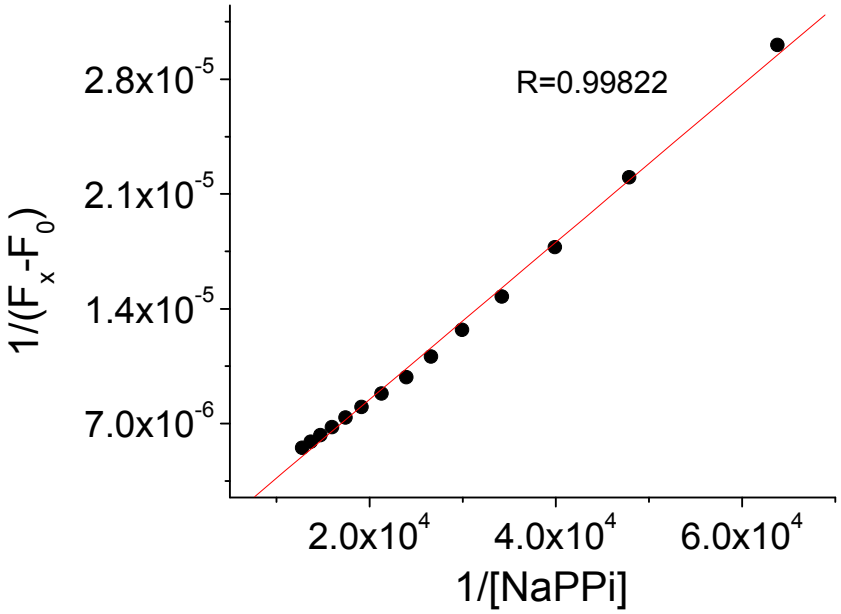
SI Figure 5: Luminescence response of **L.Cd** (2.0 x10^{-5} M) in aqueous 0.01mmol HEPES buffer (pH-7.4) medium on addition of the solution of sodium salt of various anions and nucleotides: 1. **L.Cd**, 2. NO_3^-, 3. ATP, 4. $H_2PO_4^-$, 5. I^-, 6. AMP, 7. SO_4^-, 8. PPi, 9. Cl^-, 10. Br^-, 11. $CH_3CO_2^-$, 12. ADP, 13. CTP. (2.0 x10^{-4}) with λ_{mon}=414 nm, λ_{ext} = 328 nm.

Benesi-Hildebrand plot for binding studies of NaPPI towards LZn:



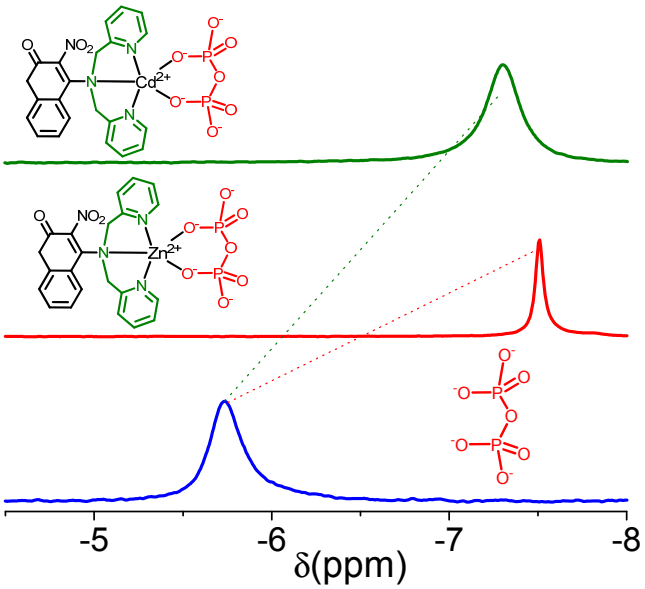
SI Figure 5: Benesi-Hildebrand plot of for evaluation of binding constant and stoichiometry for the formation of **L.Zn-PPi** complex in aqueous 0.01mmol HEPES buffer (pH-7.4) medium. λ_{ext} = 328 nm and λ_{mon} = 428 nm were used for emission studies. Goodness of the fit of the plot confirms the 1:1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

Benesi-Hildebrand plot of L.Cd with NaPPi



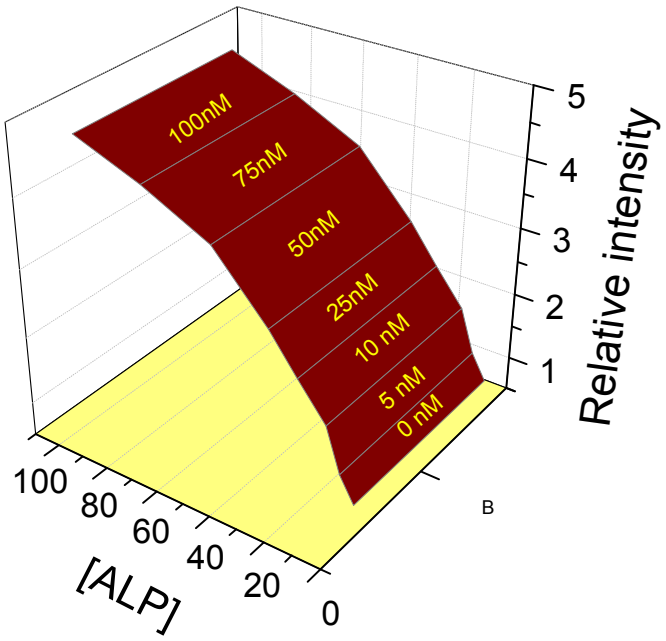
SI Figure 6: Benesi-Hildebrand plot of for evaluation of binding constant and stoichiometry for the formation of **L.Cd-PPi** complex in aqueous 0.01mmol HEPES buffer (pH-7.4) medium. λ_{ext} = 328 nm and λ_{mon} = 414 nm were used for emission studies. Goodness of the fit of the plot confirms the 1:1 binding stochiometry.

^{31}P NMR of PPi in absence and presence of L.Zn, L .Cd



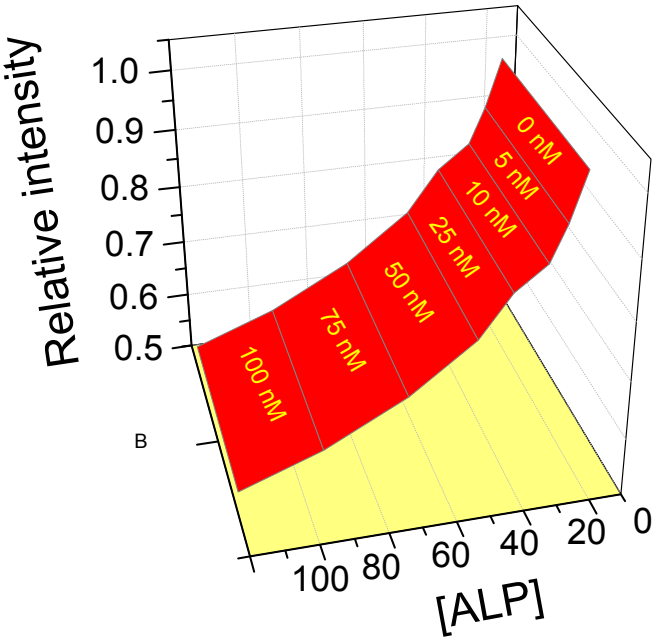
SI Figure 7: ^{31}P NMR spectra of **L.Zn** and **L.Cd** (15 mM) before and after addition of $Na_4P_2O_7$ (150 mM) in D_2O.

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



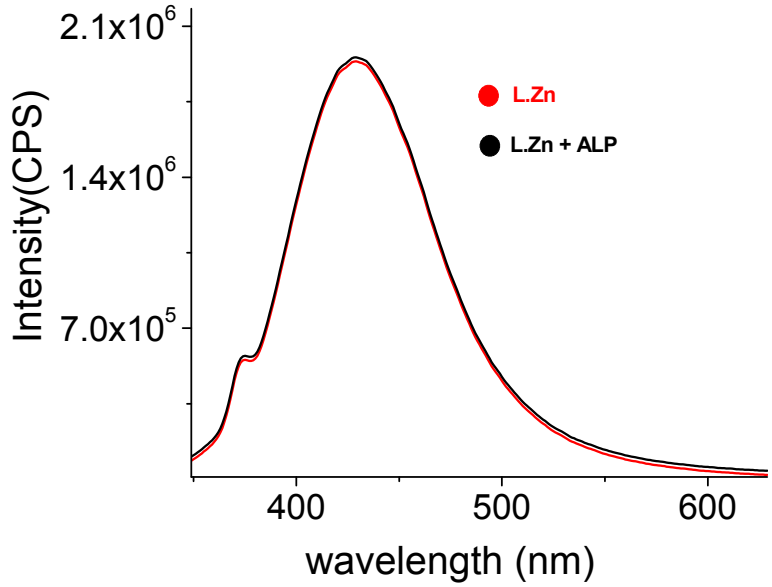
SI Figure 8: A ribbon diagram plot of relative change in emission intensity of **L.Zn** with varying [ALP] in aqueous 0.01mmol HEPES buffer (pH-7.4) medium. For each measurement of emission intensity, a time interval of 900 sec was allowed. λ_{ext} = 328 nm and λ_{mon} = 428 nm were used for emission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

A Ribbon diagram plot of Relative intensity of L.cd with varying concentration of ALP



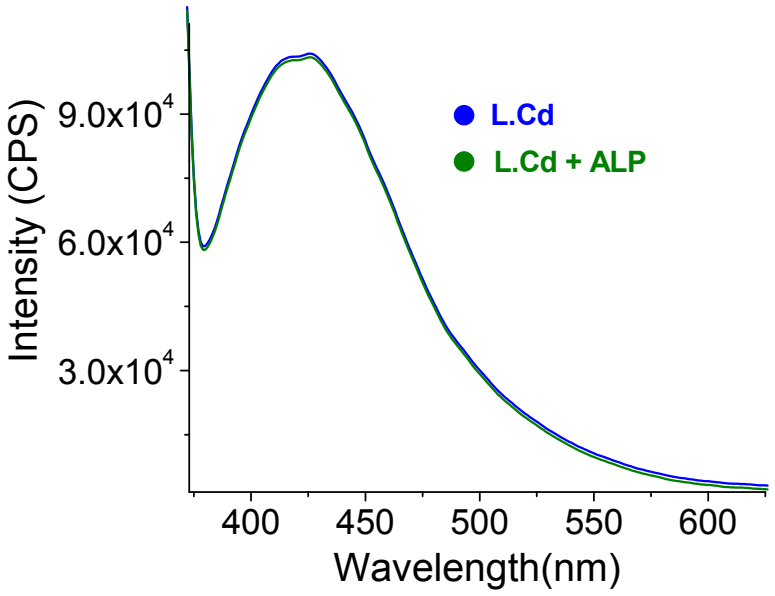
SI Figure 9: A ribbon diagram plot of relative change in emission intensity of **L.Cd** with varying [ALP] in aqueous 0.01mmol HEPES buffer (pH-7.4) medium. For each measurement of emission intensity, a time interval of 900 sec was allowed. λ_{ext} = 328 nm and λ_{mon} = 414 nm were used for emission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

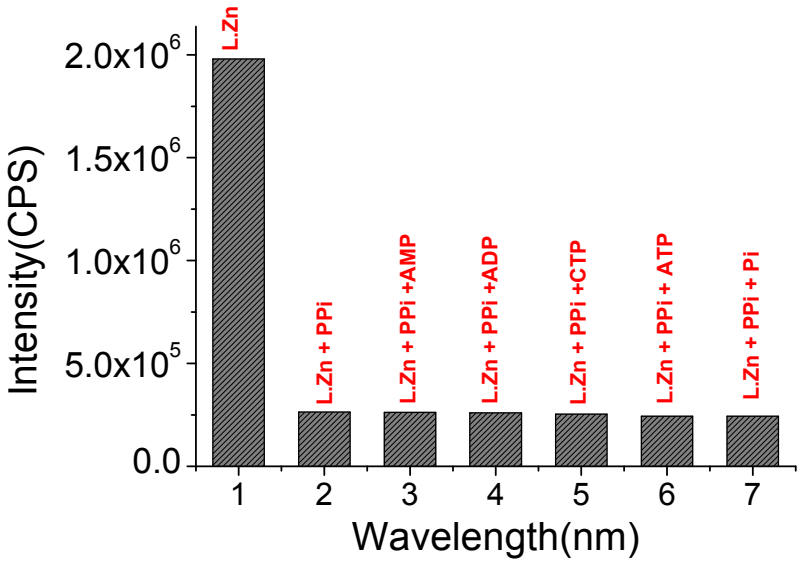
Emission spectra of LCd in absence and presence of Alkaline phosphatase



SI Figure 11: Emission spectra of **L.Cd** (2.0 $X10^{-5}$) 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 $Na_4P_2O_7$ (NaPPi) in aqueous 0.01mmol HEPES buffer (pH-7.4) medium. We had made two different types assay. First one has (i) $\mathbf{L.M^{n+}}$ ($M = Zn^{2+}$, Cd^{2+}) 2.0 $x10^{-5}$M; while the second one has (ii) $\mathbf{L.M^{n+}}$ ($M = Zn^{2+}$, Cd^{2+}) 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 $Na_4P_2O_7$ (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 $Na_4P_2O_7$ 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** (2.0×10^{-5} M) with $Na_4P_2O_7$ (1.0×10^{-4} M) in the absence and presence of 5.0×10^{-3} M of different coexisting phosphate anions and nucleotides in in aqueous 0.01mmol HEPES buffer (pH-7.4) medium (λ_{ext} = 328 nm).

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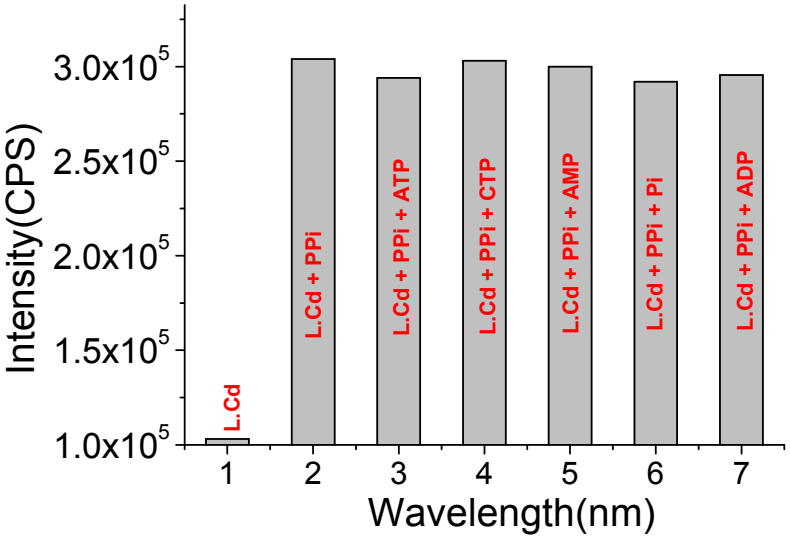
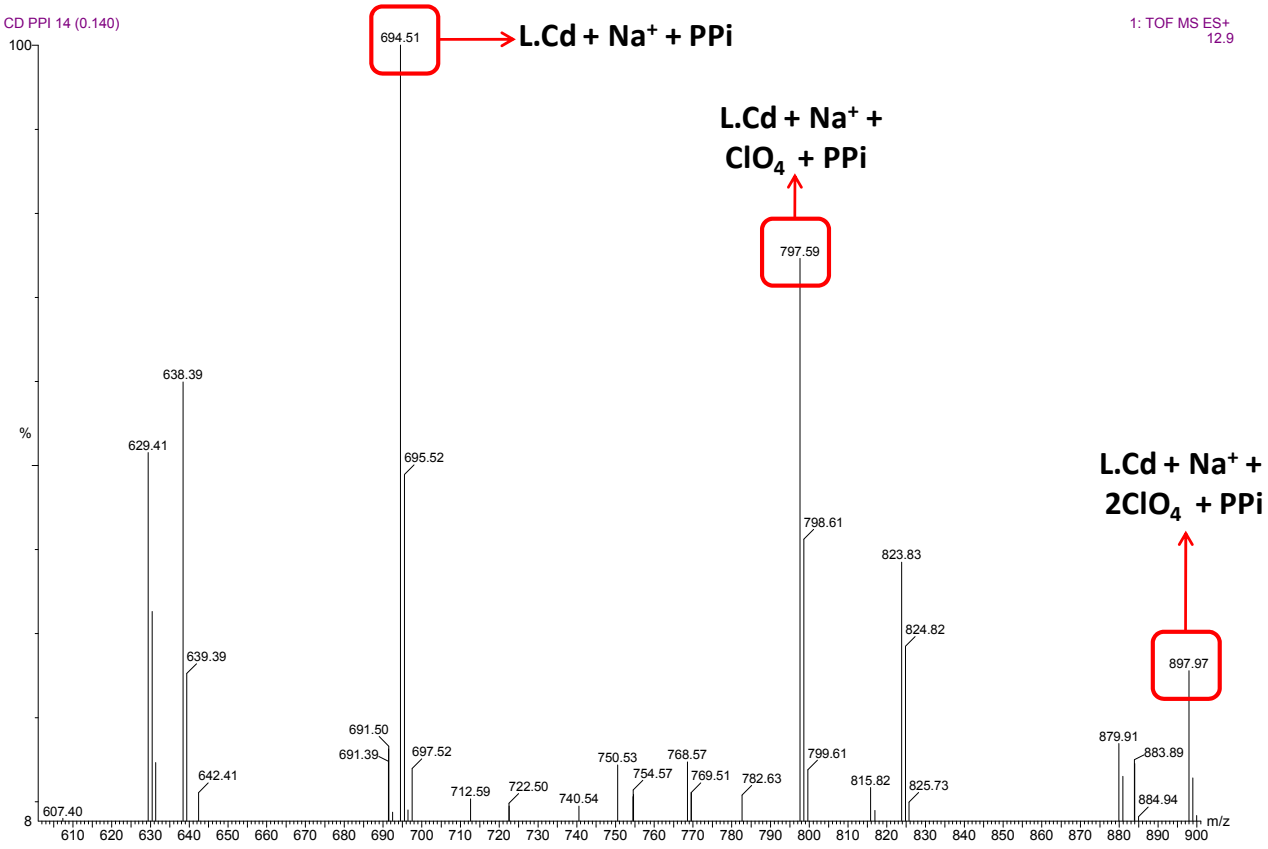
Interference Study for the binding of NaPPi with L.Cd



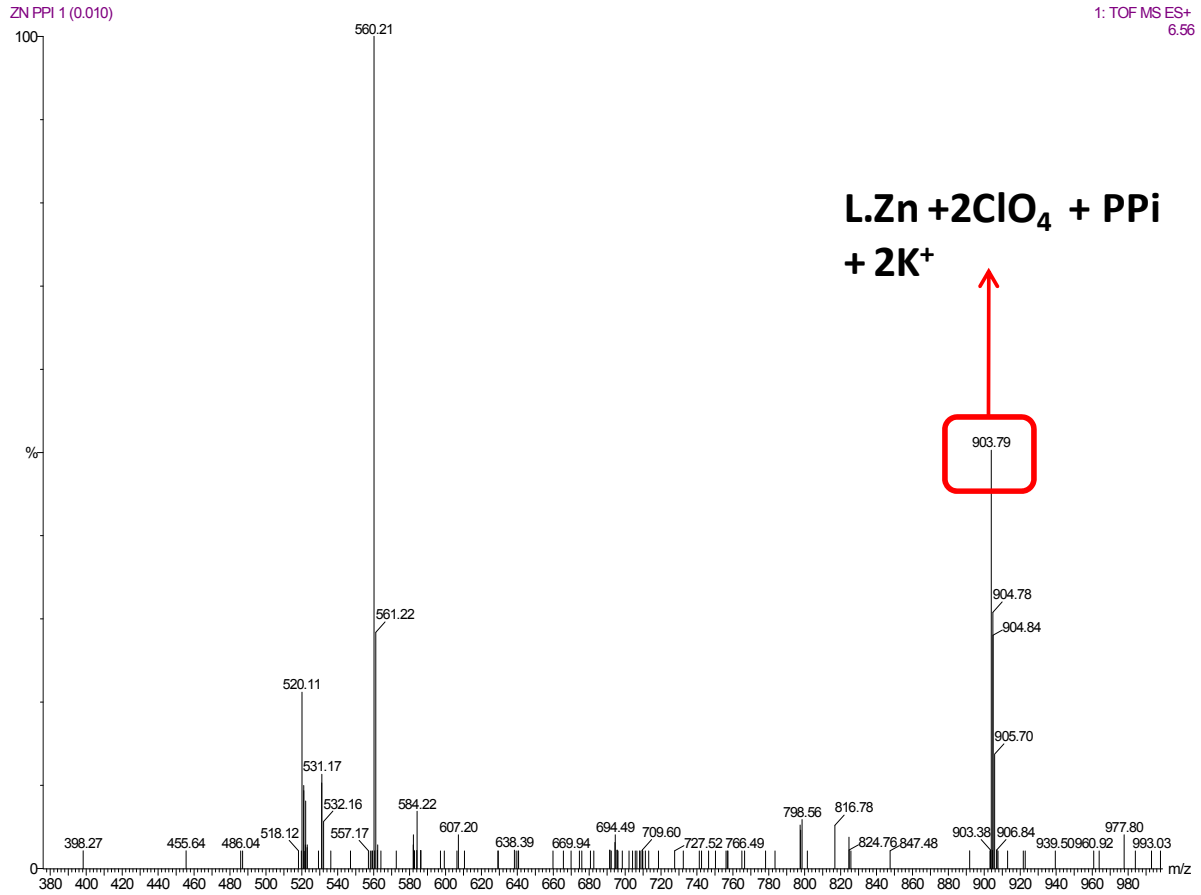
SI Figure 13: Emission intensity at 414 nm of **L.Cd** (2.0×10^{-5} M) with $Na_4P_2O_7$ (1.0×10^{-4} M) in the absence and presence of 5.0×10^{-3} M of different coexisting phosphate anions and nucleotides in in aqueous 0.01mmol HEPES buffer (pH-7.4) medium (λ_{ext} = 328 nm).

Mass spectra of L.Cd complex in presence of NaPPi

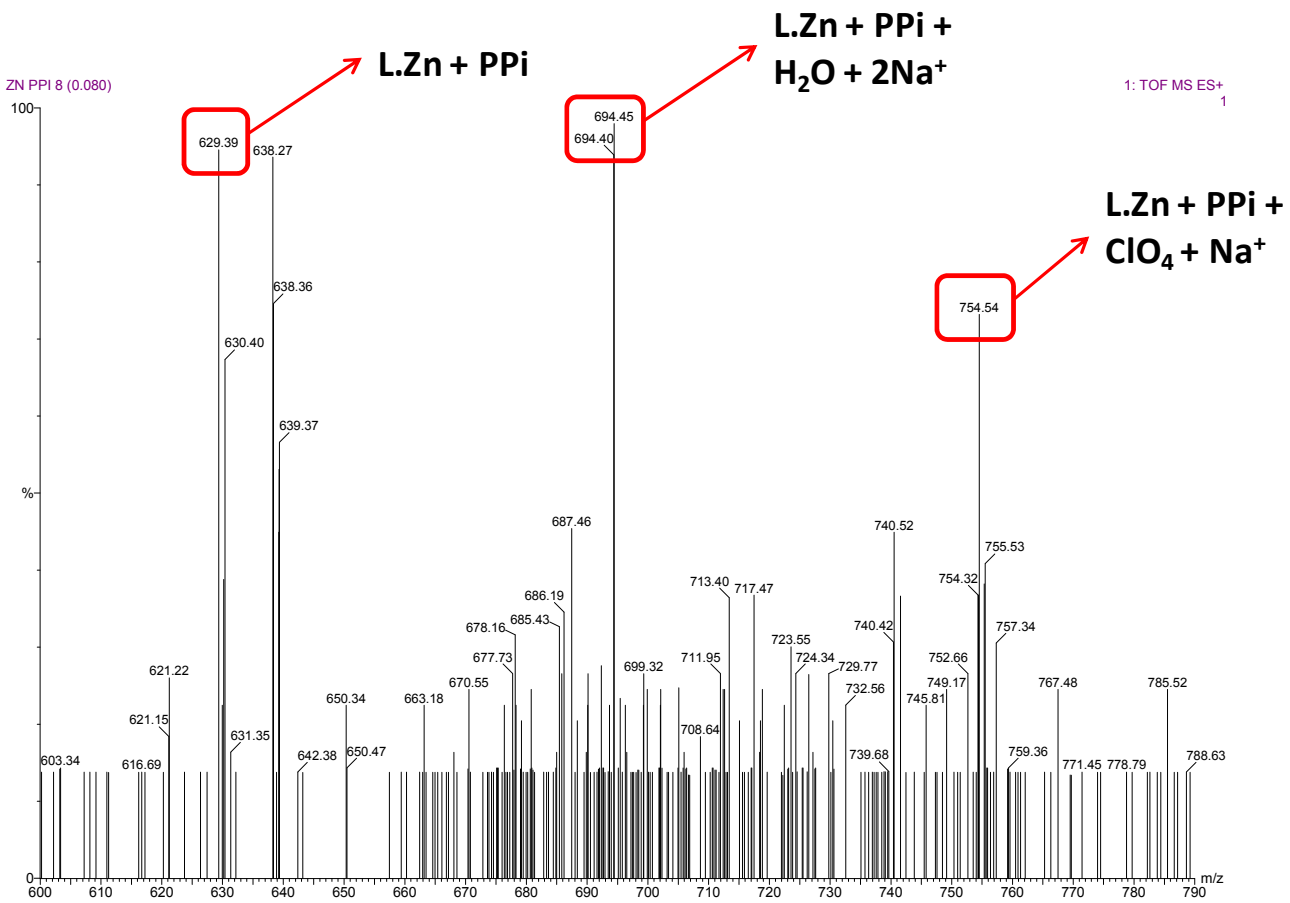


SI Figure 14: ESI-MS spectra of **L.Cd** in presence of 10 mole equivalent of NaPPi in aqueous medium.

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.

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

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

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