Supporting Information for

AIE Active Metal-Free Chemosensing Platform for Highly Selective Turn-ON Sensing and Bioimaging of Pyrophosphate Anion

Abhijit Gogoi,¹ Sandipan Mukherjee,² Aiyagari Ramesh² and Gopal Das¹

¹Department of Chemistry, Indian Institute of Technology Guwahati, Guwahati 781039, India, Fax: + 91 361 2582349; Tel: +91 3612582313; E-mail: gdas@iitg.ernet.in.
²Department of Biosciences and Bioengineering, Indian Institute of Technology, Guwahati, Guwahati, 781039, India, Fax: + 91 361 2582249; Tel: +91 3612582205; E-mail: aramesh@iitg.ernet.in.
1. **Figure S1.** Fluorescence and UV-Vis changes of L in various solvents.

2. **Figure S2.** Fluorescence emission changes of L with increasing water content in acetonitrile.

3. **Figure S3.** UV changes of L with PPI in water.

4. **Figure S4.** Job’s plot and Bensei-Hildebrand plot for PPI ion.

5. **Figure S5.** Calculation of LOD for PPI.

6. **Figure S6.** Fluorescence emission changes of L with biologically important anions.

7. **Figure S7.** Fluorescence based anion selectivity study of L in MeCN-Wat.

8. **Figure S8.** UV-Vis changes of the L with PPI in acetonitrile at room temperature.

9. **Figure S9.** Fluorescence emission change after addition of Ca$^{2+}$ to L-PPI ensemble.

10. **Figure S10.** DLS data of L with PPI.

11. **Figure S11.** MTT assay.

12. **Table S1.** Comparison of LOD values for PPI sensor.

13. **Scheme 1.** Synthetic schemes of the starting materials.

14. **Figure S12.** Mass spectrum of 4-(1H-benzo[d]imidazol-2-yl)aniline

15. **Figure S13.** $^1$H NMR of L.

16. **Figure S14.** $^{13}$C NMR of L.

17. **Figure S15.** ESI-mass spectra of L.
Experimental Section

General Information and Materials
All the materials for synthesis were purchased from commercial suppliers. The absorption spectra were recorded on a Perkin-Elmer Lamda-750 UV-Vis spectrophotometer using 10 mm path length quartz cuvettes in the range 250-700 nm wavelengths, while the fluorescence measurements were carried on a Horiba Fluoromax-4 spectrofluorometer using 10 mm path length quartz cuvettes with a slit width of 2 nm at 298 K. Mass spectrum of L was obtained using Waters Q-ToF Premier mass spectrometer. The NMR spectra were recorded on a Varian FT-400 MHz instrument and the chemical shifts are presented in parts per million (ppm) on the scale. The following abbreviations are used to describe spin multiplicities in $^1$H NMR spectra: s = singlet; d = doublet; t = triplet; m = multiplet.

Evaluation of the Apparent Binding Constant. Probe L with an effective concentration of 10µM in water was titrated with varying PPi concentration (0 and 70µM).
Thus the apparent binding constant for the formation of the respective PPi complex was evaluated using the Benesi–Hildebrand (B–H) plot (equation 1).

$$\frac{1}{(I-I_0)} = \frac{1}{K(I_{\text{max}} - I_0)C} + \frac{1}{I_{\text{max}} - I_0}$$

I$_0$ is the emission intensity of L at maximum ($\lambda = 530$ nm), I is the observed emission intensity at that particular wavelength in the presence of a certain concentration of the analyte (C), I$_{\text{max}}$ is the maximum emission intensity value that was obtained at $\lambda = 530$ nm during titration with varying analyte concentration, K is the apparent binding constant (M$^{-1}$) and was determined from the slope of the linear plot.

Detection Limit. The detection limits were calculated on the basis of the fluorescence titration changes for PPi. For the standard deviation of L, emission spectrum of the L was measured ten times. To gain the slope, the fluorescence emission at 530 nm was plotted against the concentration of PPi. The detection limits were calculated using the following equation:

$$\text{Detection limit} = 3\sigma/k$$

where $\sigma$ is the standard deviation of blank measurement, and k is the slope between the fluorescence emission intensity versus [PPi].

Dynamic light scattering studies. The particle size of the L-PPi aggregates was measured by dynamic light scattering (DLS) experiments on a Malvern Zetasizer Nano ZS instrument equipped with a 4.0 mW He–Ne laser operating at a wavelength of 633 nm. The samples and
the background were measured at room temperature (25°C) at a scattering angle of 173°. DLS experiments were carried out with an optically clear solution of L (10 μM) in water in the presence of 10 equivalents of PPi ion. The solution was equilibrated for 30 minutes prior to taking the measurements.

**Atomic Force Microscope (AFM) Studies.** Glass cover slips (18 mm x 18 mm) were sterilized by immersion in sodium hypochlorite solution (0.5%) for 2 h and rinsed thoroughly with sterile MilliQ grade water. To study the aggregation effect of L, to a solution of L (10 μM) in aqueous medium, 10 equivalents of PPi was added and mixed well. The mixture was then drop-casted on a glass cover slip followed by dessication prior to acquiring AFM images. Atomic force microscope images were captured with an Agilent 5500 AFM (Agilent Technologies, Chandler, AZ, USA). Cantilevers made of silicon nitride were used having a resonant frequency of ca. 150 to 250 kHz. Images were acquired in non-contact mode, with 10 μm x 10 μm size at a scan rate of 0.5-1.0 line/s. Analysis of the topographic images of the surface was accomplished by using the WSxM v5.0 Develop 6.5 image viewer software.

**Field Emission Scanning Electron Microscope (FESEM) Studies.** FESEM imaging studies were conducted separately with (i) a solution of L (10 μM) in DMSO, (ii) an aqueous solution of L (10 μM) and (iii) an aqueous solution of L (10 μM) mixed with 10 equivalents of PPi. The solutions were then drop-casted on a cover slip, followed by dessication prior to acquiring images in a field emission scanning electron microscope (Zeiss Sigma, USA).

**Cytotoxic Effect on HeLa Cells.** The cytotoxic effect of the probe and probe-PPi complex on cultured HeLa cells was determined by an MTT assay as per the manufacturer instruction (Sigma-Aldrich, MO, USA). HeLa cells were initially grown in a 25 cm² tissue culture flask in DMEM medium supplemented with 10% (v/v) FBS, penicillin (100 μg/mL) and streptomycin (100 μg/mL) under a humidified atmosphere of 5% CO₂ in an incubator until the cells were approximately 90% confluent. Prior to MTT assay, cells were trypsinized and seeded into 96 well tissue culture plates at a cell-density of 10⁴ cells per well and incubated with varying concentrations (10, 20, 30, 40, 50 and 60 μM) of the probe, probe-PPi complex and the metal salt solution made in methanol solvent (0.1% v/v) and incubated for a period of 24 h under 5% CO₂. Solvent control samples (cells incubated in 0.1% methanol) were also included in parallel sets. Following incubation, the growth media was carefully aspirated, and fresh DMEM containing MTT solution was added to the wells. The plate was incubated for 4 h at 37°C. Following incubation, the supernatant was collected and the insoluble colored formazan product was solubilized in DMSO and its absorbance was measured in a microtiter
plate reader (Infinite M200, TECAN, Switzerland) at 550 nm. The assay was performed in six sets for each concentration of the test samples. Data analysis and determination of standard deviation was performed with Microsoft Excel 2010 (Microsoft Corporation). In the MTT assay, the absorbance for the solvent control cells was considered as 100% cell viability and the absorbance for the treated cells was compared to determine % cell viability with respect to the solvent control.

Figure S1. A) Fluorescence and B) UV-Vis changes of L (10 μM) in various solvents at room temperature.
**Figure S2.** Fluorescence emission changes of L with increasing water content in acetonitrile solution ($\lambda_{\text{ex}} = 430$ nm, slit = 2nm / 2nm).

**Figure S3.** UV changes of L with a) various anions (10 equv.) and b) magnified view with PPI in water. Inset: Change in the color of L after PPI addition.
Figure S4. A) Job’s plot for determining the stoichiometry of L with PPi (1:1 host-guest complex). B) Bensei-Hildebrand plot for PPi ion.

Figure S5. Fluorescence emission intensity at 530 nm of L against pyrophosphate (PPi) concentrations for calculation of limit of detection (LOD).
**Figure S6.** Fluorescence intensity changes of L with the some biologically important anions in water.

**Figure S7.** Fluorescence based anion selectivity study of L in MeCN-water (1:1, v/v) mix at room temperature ($\lambda_{ex} = 430$ nm, slit = 2nm / 2nm).
**Figure S8.** UV-Vis changes of the L (10 μM) with PPi (10 equiv.) in dry acetonitrile at room temperature.

**Figure S9.** Fluorescence emission changes ‘L-PPi’ ensemble after addition of Ca$^{2+}$ ($\lambda_{ex} = 430$ nm, slit = 2nm/2nm).
Figure S10. DLS analysis of L (10 μM) after addition of PPI (10 equiv.) in water.

Figure S11. MTT based cytotoxicity assay for ligand and L-PPI ensemble.
**Table S1.** Recent progress in the field of PPi sensing, a comparative study of their LOD values in different binding environments.

<table>
<thead>
<tr>
<th>Sl No.</th>
<th>References</th>
<th>Used system</th>
<th>LOD of PPI</th>
<th>Solvent system</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Present work</td>
<td>Probe alone</td>
<td>1.67 nM</td>
<td>Water</td>
</tr>
<tr>
<td>2.</td>
<td>J. Am. Chem. Soc. 2014, 136, 5543</td>
<td>Probe-Zn(II) ensemble</td>
<td>0.8 nM</td>
<td>10 mM HEPES buffer (pH 7.4)</td>
</tr>
<tr>
<td>3.</td>
<td>RSC Adv., 2015, 5, 10505–10511</td>
<td>Probe-Zn(II) ensemble</td>
<td>7.27 ×10⁻⁷ M</td>
<td>10 mM, HEPES 10 mM, pH = 7.4</td>
</tr>
<tr>
<td>5.</td>
<td>Chem. Commun., 2012, 48, 1784–1786</td>
<td>Probe-Cu(II) ensemble</td>
<td>2.02 mM</td>
<td>Aqueous solution buffered with MOPS (10 mM, pH = 7.0)</td>
</tr>
<tr>
<td>6.</td>
<td>J. Org. Chem. 2012, 77, 11405–11408</td>
<td>Probe-Zn(II) ensemble</td>
<td>Not reported</td>
<td>Aqueous solution of 50 mM HEPES buffer (pH = 7.4)</td>
</tr>
<tr>
<td>7.</td>
<td>Inorg. Chem., 2013, 52 (15), 8294–8296</td>
<td>Probe-Zn(II) ensemble</td>
<td>155 × 10⁻⁸ M</td>
<td>0.02 M HEPES buffer pH = 7.4</td>
</tr>
<tr>
<td>8.</td>
<td>Org. Lett. 2014, 16, 2220–2223</td>
<td>Probe-Cu(II) ensemble</td>
<td>Not reported</td>
<td>HEPES buffer (10 mM, pH 7.0)</td>
</tr>
<tr>
<td>9.</td>
<td>Dalton Trans., 2015,44, 1358–1365</td>
<td>Probe-Cd(II) ensemble</td>
<td>3.39 μM</td>
<td>CH₃CN/HEPES(10mM, pH = 7.4) = 1:1(v:v),</td>
</tr>
<tr>
<td>10.</td>
<td>RSC Adv., 2014, 4, 484</td>
<td>Probe-Zn(II) ensemble</td>
<td>Not reported</td>
<td>10 mM aqueous HEPES buffer (pH 7.2) at 25°C</td>
</tr>
<tr>
<td>11.</td>
<td>Org. Lett., 2011, 13, 5294</td>
<td>Probe-Zn(II) ensemble</td>
<td>1.5 μM</td>
<td>CH₃CN/20 mM HEPES=5: 95, pH 7.4</td>
</tr>
<tr>
<td>12.</td>
<td>Anal.Chem,2010, 82, 4628-4636</td>
<td>Probe-Zn(II) ensemble</td>
<td>0.4 μM</td>
<td>30% ethanol/water (v/v) solution at pH 7.4</td>
</tr>
</tbody>
</table>

**Table S2.** Fluorescence quantum yields (QY) of L in solution and solid state.

<table>
<thead>
<tr>
<th></th>
<th>L in THF</th>
<th>L in MeOH</th>
<th>L in H₂O</th>
<th>L-PPi in H₂O</th>
<th>powder</th>
</tr>
</thead>
<tbody>
<tr>
<td>QY</td>
<td>8.5 %</td>
<td>17.3 %</td>
<td>27.5 %</td>
<td>43.6 %</td>
<td>18.8 %</td>
</tr>
</tbody>
</table>
Synthesis of 4-(1H-benzo[d]imidazol-2-yl)aniline: 4-aminobenzoic acid and o-phenylenediamine were dissolved in 20 ml of 4N HCl and refluxed with stirring at 170°C for 24 hrs. After that the solution was neutralized with sodium bicarbonate solution and then extracted with CHCl₃. Combined organic phase was washed with brined and dried (MgSO₄) which gives off brown solid. Crystallization in methanol finally gives the desired product. Yield: 65%. M+H⁺ = 210.25, found = 210.1071. ¹H NMR: 7.853–7.834 (d, 2H), 7.473 (2H, bs), 7.101(bs, 2H), 6.771–6.653(d, 2H), 5.591(s, 2H).

Scheme S1. Synthetic schemes of the starting materials.

Figure S12. ESI-mass spectra of 4-(1H-benzo[d]imidazol-2-yl)aniline in positive ionization mode.
2,6-Diformyl-4-methylphenol: It was synthesized according to the reported literature procedure.²

![Figure S13. ¹H NMR of L in DMSO-d₆ at room temperature.](image1)

![Figure S14. ¹³C NMR of L in DMSO-d₆ at room temperature.](image2)
Figure S15. ESI-mass spectra of L.

References:
