Mercury is considered a neurotoxin among heavy metal ions, because of its high affinity towards thiols and amino groups in peptides and nucleic acids, resulting in many health disorders, such as kidney failure, myocardial infarction, brain damage and Minamata disease. The development of new technologies and analytical methods for selective and accurate detection of Hg²⁺ ions is still a growing research field. Over the past few years, fluorometry has appeared as a useful technique for the selective and sensitive detection of various analytes, such as metal ion, anions, peptides, nucleotides and amino acids, because of its high affinity towards thiols and amino groups in analytes. Interestingly, the non-fluorescent complex \([\text{Hg(LPy)}_2]\) is able to specifically respond towards Cys over other biothiols and amino acids through a reversible de-complexation mechanism. As a result, the remarkable recovery of the fluorescence can be observed. The limit of detection (LOD) for Cys detection is estimated to be 29 nM in DMF:H₂O (1:9 v/v, 10 mM HEPES buffer, pH 8.0). The reversibility and reusability of \([\text{Hg(LPy)}_2]\) were achieved by the sequential addition of Cys and Hg²⁺ ions up to five cycles. Moreover, the removal of Hg²⁺ ions up to 89% from aqueous samples using HL₉ was successfully demonstrated.

**Keywords** BODIPY, Hg²⁺ ions detection, cysteine, Job’s plot

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HLPy the removal of Hg\(\text{II}\) ions from real water samples. \([\text{Hg}(\text{L Py})_2]\) plausible sensing mechanism.

by routine procedures before use.\(^47,48\) The precursor compounds

Materials and instrumentation

Experimental

Synthesis of BODIPY-Hg\(\text{II}\) complex \([\text{Hg}(\text{L Py})_2]\).

synthesized by the reaction of Hg(OAc)\(_2\) to the DMF solution of HLPy in the stoichiometric amount, as presented in Scheme 1. As intended, the resulting non-fluorescent complex \([\text{Hg}(\text{L Py})_2]\) demonstrated a "Turn-ON" fluorescence specifically to Cys over other biothiols and amino acids through the de-complexation mechanism. In this report, we have presented a comprehensive study of sensing properties in both \textit{in-situ} (BODIPY-Hg\(\text{II}\)) and isolated complex \([\text{Hg}(\text{L Py})_2]\) material. To the extent of our knowledge, this is the first report of its kind, where a BODIPY-Hg\(\text{II}\) complex \([\text{Hg}(\text{L Py})_2]\) has been synthesized and evaluated for the selective detection of Cys. Most of the previous reports are based on the \textit{in-situ} generated complex species only. Some of the recently reported BODIPY based sensing probes and their sensing properties have been summarized and compared with \([\text{Hg}(\text{L Py})_2]\) in Table 1.\(^35–44\) Further, the reversibility and reusability of the \([\text{Hg}(\text{L Py})_2]\) for the detection of Cys were confirmed up to five cycles and also demonstrated through a logic gate system. The utility of HLPy was also established in the removal of Hg\(\text{II}\) ions from real water samples.

Experimental

Materials and instrumentation

All reagents and solvents of analytical grade were purchased from commercial suppliers. Solvents were dried and distilled by routine procedures before use.\(^45,46\) The precursor compounds 1–3 were synthesized using the modified reported procedures, details of which are provided in Scheme S1 (Supporting Information). The synthesized compounds were analyzed by several spectroscopic and analysis techniques. The absorption and fluorescence spectral measurements were recorded on an Agilent Carry 100 spectrophotometer and LS55 fluorescence spectrometer, respectively, with 1.0 cm path length quartz cuvette at ambient temperature with an excitation slit width of 5 nm. \(^1\)H NMR, \(^13\)C NMR, \(^19\)F NMR and \(^11\)B NMR spectra were recorded in DMSO-d\(_6\) and CDCl\(_3\) on a Jeol 400 MHz instrument. The chemical shifts were reported in \(\delta\) ppm with coupling constant (\(J\)) in Hz. ESI mass spectra were obtained by using an ABSciex TOF/TOF 5800 system. FT-IR (Zn-Se, ATR) spectra were recorded on a Bruker Alpha spectrophotometer. Elemental analysis data were obtained by Netzsch 200F3. All pH measurements were carried out with an Oakton 35630-90 pH meter. Thermogravimetric analysis (TGA) was conducted on a Perkin Elmer TGA4000 instrument with a heating rate of 10°C min\(^{-1}\). Differential scanning calorimetry (DSC) was performed on a Perkin Elmer DSC6000 instrument with a heating rate of 10°C min\(^{-1}\). Single-crystal X-ray diffraction data for 3 and HLPy were collected on a charge-coupled-device (CCD) diffractometer with graphite-monochromatized Mo K\(\alpha\) X-ray radiation (\(\lambda = 0.71073\) Å).\(^49,50\) The crystallographic data were solved by direct methods and refined by full-matrix least-squares against \(F^2\) using the program SHELXL-97 in WinGX module.\(^51\) Non-hydrogen atoms were refined anisotropically. Details of crystallographic data and structure refinement parameters for 3 and HLPy are given in Table S1 (Supporting Information).

Absorption and fluorescence spectroscopic studies

The aqueous stock solutions (5 mM) of metal nitrate salts of Co\(\text{II}\), Ca\(\text{II}\), Cd\(\text{II}\), Ag\(\text{I}\), Cr\(\text{III}\), Cu\(\text{II}\), K\(\text{I}\), Fe\(\text{III}\), Hg\(\text{II}\), Ni\(\text{II}\), Pb\(\text{II}\), Zn\(\text{II}\), Al\(\text{III}\), Na\(\text{I}\) and Mn\(\text{II}\) were prepared in deionized water. All the photophysical studies of HLPy were performed with 10 mM HEPES buffer at pH 7.4. The working solution (10 μM) of HLPy was prepared in DMF:H\(_2\)O (1:9 v/v, 10 mM HEPES buffer, pH 7.4). The stock solution (5 mM) of biologically relevant analytes such as cysteine (Cys), homocysteine (Hcy), methionine (Met), glutathione (GSH), alanine (Ala), tryptophan (Trp), histidine (His) and proline (Pro) were also prepared in deionized water. Based on the solubility criteria of synthesized non-fluorescent BODIPY-Hg\(\text{II}\) complex \([\text{Hg}(\text{L Py})_2]\), the effect of various amino acids and biothiols on its photophysical properties were examined in DMF:H\(_2\)O (1:9 v/v, 10 mM HEPES buffer, pH 8.0) solvent system using 10 μM working solution of the complex. For all spectroscopic measurements, the excitation wavelength taken for HLPy and its complex \([\text{Hg}(\text{L Py})_2]\) was 450 and 480 nm, respectively, with slit width 5 nm (unless noted otherwise). The sample size is three for all spectroscopic measurements taken during the experiments.

Calculation of association constant, fluorescence quantum yields and LOD

The association constant (\(K_a\)) and limit of detection (LOD) were estimated on the basis of emission titration studies of HLPy and \([\text{Hg}(\text{L Py})_2]\) with an effective concentration of 10 μM, using Benesi-Hildebrand equation (Eq. (1)).\(^52\) The effective concentrations of Hg\(\text{II}\) varied between 0 and 5 μM for titration with HLPy, whereas the Cys concentrations changed between 0 and 20 μM for titration with \([\text{Hg}(\text{L Py})_2]\).

\[
\frac{1}{(I - I_0)} = \frac{1}{K_a(I_0 - I)[\text{M}^+] + 1/(I_0 - I)},
\]

where \(I_0\) represents the emission intensity of HLPy and \([\text{Hg}(\text{L Py})_2]\) at \(\lambda = 567\) and 575 nm, respectively. \(I\) represents the observed emission intensity of HLPy and \([\text{Hg}(\text{L Py})_2]\) at their respective \(\lambda_{em}\) in the presence of a certain concentration of Hg\(\text{II}\) ions and biothiols, respectively.

The LOD was determined using the following equation (Eq. (2)).\(^53\)

\[
\text{Limit of detection (LOD)} = 3\sigma/k,
\]

where \(\sigma\) is the standard deviation (SD) and \(k\) is the slope of the calibration line.

The Stern-Volmer constants (\(K_{SV}\)) were calculated using Stern-Volmer equation (Eq. (3)).\(^54\)
A comparison of recently reported BODIPY based fluorescent probes with $[\text{Hg}(L^p)_2]$ in Cys detection

| S. No. | Fluorescent probe | Medium                         | LOD for Cys/M | Interference                  | Ref. |
|--------|-------------------|--------------------------------|---------------|-------------------------------|------|
| 1      |                   | CH$_3$OH:H$_2$O (4:1 v/v, pH 7.4) | $7.2 \times 10^{-6}$ | 3-Mercaptopropionic acid, homocysteine | 35   |
| 2      |                   | HEPES buffer (1% CH$_3$CN, pH 7.4) | $1 \times 10^{-7}$ | None                           | 36   |
| 3      |                   | HEPES buffer (1% CH$_3$CN, pH 7.4) | $8 \times 10^{-7}$ | Homocysteine                   | 37   |
| 4      |                   | CH$_3$OH:HEPES (1:1 v/v, pH 7.2)  | $3.3 \times 10^{-6}$ | Homocysteine                   | 38   |
| 5      |                   | PBS buffer (20% CH$_3$CN, pH 7.4) | $3 \times 10^{-6}$ | Homocysteine                   | 39   |
| 6      |                   | CH$_3$CN:PBS (1:1 v/v, pH 7.4)    | $5.1 \times 10^{-4}$ | No                             | 40   |
| 7      |                   | CH$_3$CN:H$_2$O (1:1 v/v, pH 7.4) | $1.7 \times 10^{-7}$ | Homocysteine                   | 41   |
| 8      |                   | CH$_3$CN-H$_2$O                  | $1.6 \times 10^{-8}$ | No                             | 42   |
| 9      |                   | CH$_3$OH:HEPES (30:70 v/v, pH 6.5) | $6 \times 10^{-4}$ | No                             | 43   |
| 10     |                   | CH$_3$OH:HEPES (1:1 v/v, pH 7.4)  | —              | Homocysteine, glutathione     | 44   |
| 11     |                   | DMF:H$_2$O (1:9 v/v, 10 mM HEPES buffer, pH 8.0) | $2.9 \times 10^{-4}$ | No                             | This work |
Similarly, the effect of pH on the detection has been evaluated ε on the “Turn-ON” fluorescence behavior of known quantum yield.

\[
\Phi = \Phi_0 (I/I_0) (A/A_0)
\]

Optimization of experimental parameters for biothiol detection

Systematic optimization of experimental conditions has been carried out in various solvent systems and a wide pH range (i.e., 2.0 – 10). The absorption and emission properties of both HLPy and its complex [Hg(LPy)2] were analyzed in different solvents (Figs. S18 and S19, Supporting Information). The detailed photophysical data are presented in Table S2 (Supporting Information). The absorption data reveals that HLPy has a higher molar extinction coefficient (ε > 79000 M⁻¹ cm⁻¹ at 488 nm) in DMF. Further, based upon the solubility and photophysical responses of [Hg(LPy)2], a common solvent system DMF:H₂O (1:9 v/v) was used for all measurements. Similarly, the effect of pH on the detection has been evaluated by using different buffer solutions (CH₃COOH/CH₃COONa, NaHPO₄/Na₂PO₄, NH₃/NH₄⁺ and HEPES). The emission spectra of [Hg(LPy)2] (10 μM solution in DMF:buffer) were recorded upon addition of Cys (20 μM in H₂O) in the pH range 2.0 – 10.0. No significant effect of pH in the range of 4.0 – 10 pH was observed on the “Turn-ON” fluorescence behavior of [Hg(LPy)2], indicating that synthesized [Hg(LPy)2] acts as a chemosensor at physiological pH. However, the maximum emission intensity was recovered at pH 8.0, suggesting it to be a suitable pH for further studies (Fig. S32, Supporting Information).

Synthesis and characterization of HLPy

The precursor compound 3 (0.5 g, 1.26 mmol) was treated with 2-amino pyridine (0.06 g, 0.63 mmol) in THF at ambient temperature with continuous stirring, as shown in Scheme S1. The progress of the reaction was monitored by thin-layer chromatography (TLC) at regular intervals. A prominent bright red fluorescent spot that appeared on TLC corresponded to the desired 2-amino pyridine substituted HLPy and the reaction reached completion in 12 h. The volatiles were removed under reduced pressure to yield a red colored crude product, which was purified by column chromatography with ethylacetate and hexane (20:80) to produce a pure red colored powder of HLPy. Further purification of HLPy was done by crystallization in ethyl acetate and hexane solution under slow evaporation method, resulting in a good quality crystalline product within 2 – 3 days, suitable for single crystal X-ray analysis. The product was isolated by filtration and dried under vacuum. Yield: 0.4 g, (70%). Mp 223.9°C. Anal. Calcd. for C₂₂H₁₆BClF₂N₄O₂ (452.10): C, 47.87; H, 2.74; N, 10.15. Found: C, 47.67; H, 2.95; N, 10.27.

1H NMR (400 MHz, CDCl₃, δ in ppm): 7.86 (d, 1H, CH₂), 7.79 (d, 1H, CH₂), 7.16 (t, 1H, CH₃), 7.08 (d, 1H, CH₂), 7.02 (d, 1H, CH₃), 6.53 (d, 1H, CH₂), 6.37 (d, 1H, CH₂), 4.08 (s, 3H, –CH₂). ¹³C NMR (100 MHz, DMSO-d₆, δ in ppm): 139.28, 138.09, 135.71, 128.26, 121.67, 131.67, 132.02, 119.82, 129.64, 121.02, 120.78, 118.51, 116.41, 113.84, 52.92. ¹B NMR (100 MHz, DMSO-d₆, δ in ppm): –0.25 (t, 1J(B-F), 1B). ¹⁹F NMR (300 MHz, DMSO-d₆, δ in ppm): –143.60 (q, ¹J(B-F), 2F). ESI-MS: m/z calculated for C₂₂H₁₆BClF₂N₄O₂ [M] = 452.10, found [M+H⁺] = 453.1. λₐbs (nm) in DMF = 488. λₐem (nm) in DMF = 565.

Results and Discussion

Synthesis and properties of HLPy and its complex [Hg(LPy)2] complex

The desired α-substituted BODIPY based fluorescent ligand HLPy was prepared through multi-step reactions as outlined in Scheme S1. The reaction of the stoichiometric amount of 2-amino pyridine with precursor compound 3 in THF under inert atmosphere produces fluorophore HLPy in decent yield. The single crystals suitable for X-ray analysis of precursor molecule 3 and HLPy were grown from the slow evaporation of concentrated ethyl acetate and hexane solution. The precursor compounds 1 – 3 and HLPy were well characterized by using various spectroscopic techniques and analyses. Details are given in Figs. S1 – S9 (Supporting Information). The molecular structure of compounds 3 (Fig. S10, Supporting Information) and HLPy (Fig. 1) were determined by single-crystal X-ray analysis. The X-ray crystal structure of HLPy reveals that one of the chloride atoms of 3 was substituted by 2-amino pyridine moiety to produce asymmetrical α-substituted BODIPY based fluorophore HLPy. Both the BODIPY core and the 2-amino pyridine are in the same plane to enable effective electronic communication between them.

The metal-binding ability of HLPy (10 μM) was evaluated by qualitative addition of 5 μM of various mono, di and trivalent metal ions in DMF:H₂O (1:9 v/v, 10 mM HEPES buffer, pH 7.4). Interestingly, no significant changes in the photophysical
properties were observed upon the addition of any of the metal ions, except Hg$^{2+}$. An immediate color change from orange to deep pink was found only on the addition of the Hg$^{2+}$ ion; no other metal ion showed any significant color change upon addition to the HL$^p$ solution (Fig. S20, Supporting Information). The difference in the color after the addition of Hg$^{2+}$ was further confirmed by recording UV-visible spectra (Fig. S21, Supporting Information). The results obtained from the absorption study encouraged us to explore the utility of HL$^p$ as a selective and sensitive chemosensor for mercury ion detection (vide infra as well as the metal complexation reaction between HL$^p$ with Hg$^{2+}$ ion. To determine the binding stoichiometry, emission titration experiments (Job’s plot) in the presence of varying mole-fractions of Hg$^{2+}$ in DMF:H$_2$O (1:9 v/v, 10 mM HEPES buffer, pH 7.4) were performed, which revealed 2:1 binding stoichiometry between HL$^p$ and Hg$^{2+}$ ion (Fig. S26, Supporting Information). Based on preliminary results as mentioned above, the complexation reaction was performed by the addition of HL$^p$ with Hg(OAc)$_2$ in stoichiometric amount (as suggested by Job’s plot) in DMF, resulting in the formation of a non-fluorescent [Hg(L$^p$)$_2$] complex as shown in Scheme 1. The synthesized non-fluorescent [Hg(L$^p$)$_2$] was well characterized by using various spectroscopic techniques and the results were compared with the fluorescent parent molecule HL$^p$. In FT-IR spectra of HL$^p$ (Fig. S1) the vN-H stretching$^9$ observed at 3376 cm$^{-1}$ was missing in the FT-IR of [Hg(L$^p$)$_2$], which indicates that the Hg$^{2+}$ ion coordinated to HL$^p$ through the deprotonated amine nitrogen (Fig. S11, Supporting Information). The comparative $^1$H NMR spectra of [Hg(L$^p$)$_2$] and HL$^p$ were recorded in DMSO-d$_6$ (Fig. 2a) and also support the findings of FT-IR. The N-H signal at 10.08 ppm for HL$^p$ disappeared in $^1$H NMR of [Hg(L$^p$)$_2$]. Importantly, the $^1$H NMR of [Hg(L$^p$)$_2$] shows two close singlets in the aliphatic region at 3.9 and 3.7 ppm corresponding to three protons each of two different –CH$_3$ groups (Fig. 2a and Fig. S12, Supporting Information). As expected, quite broad features in the aromatic region (6 - 8.5 ppm) were obtained in $^1$H NMR spectra of [Hg(L$^p$)$_2$] corresponding to 24 aromatic protons, which clearly suggests that two deprotonated ligands were coordinated with Hg$^{2+}$ to yield 2:1 stoichiometric BODIPY-Hg$^{2+}$ complex [Hg(L$^p$)$_2$]. Also, the appearances of two different signals for –CH$_3$ groups in $^1$H NMR recorded in DMSO-d$_6$ suggest that both coordinated ligands are in an asymmetrical electronic environment. However, to better understand the asymmetrical nature of [Hg(L$^p$)$_2$], the $^1$H NMR was also recorded in CDCl$_3$, resulting in one un-split feature for six aliphatic –CH$_3$ protons (Fig. S13, Supporting Information). This clearly indicates that the asymmetrical nature of [Hg(L$^p$)$_2$] is medium dependent and might be observed only in high polar solvents. Further, the comparative $^1$B and $^19$F NMR of HL$^p$ and [Hg(L$^p$)$_2$] are shown in Figs. 2b and 2c, respectively. The $^19$F NMR of the complex shows two resonance quadrat features at $-$139.50 and $-$143.30 ppm (Fig. S14, Supporting Information), suggesting that both F atoms attached to the boron are influenced by the Hg$^{2+}$ unequally and may produce the asymmetrical electronic environment. A comparatively broad triplet signal at $-$0.19, slightly downfield from the free ligand (–0.25) was observed in $^1$B NMR of [Hg(L$^p$)$_2$] due to shifting of electron density towards the metal center as shown in Supporting Information (Figs. S8 and S15). The molecular ion peak at $m/z$ =1104.33 was obtained in HRMS spectra, which confirms the formation of 2:1 stoichiometric complex of HL$^p$ with Hg$^{2+}$ ions (Fig. S16, Supporting Information). Additionally, the observed isotopic peak pattern has been found to be in excellent agreement with those theoretically calculated for the dimeric complex. The thermal stability of both HL$^p$ and [Hg(L$^p$)$_2$] was examined by recording thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC). The TGA analysis showed excellent thermal stability of [Hg(L$^p$)$_2$] up to 255°C. Much greater decomposition of [Hg(L$^p$)$_2$] in the temperature range of 255 - 450°C corresponds to the removal of Hg in the form of HgO (Fig. S17a, Supporting Information) with an extra weight loss of 28.5% as compared to HL$^p$. The DSC spectra suggest that both HL$^p$ and [Hg(L$^p$)$_2$] have quite good thermal

Fig. 1 Molecular structure of HL$^p$. Thermal ellipsoids are drawn at 50% probability label.

Fig. 2 Comparative $^1$H NMR (a), $^1$B NMR (b) and $^19$F NMR (c) of HL$^p$ and [Hg(L$^p$)$_2$] recorded in DMSO-d$_6$. 
Conductivity measurement data reveals the non-electrolytic nature of \([\text{Hg}(\text{LPy})_2]\) in DMF. Elemental analysis of \([\text{Hg}(\text{LPy})_2]\) unambiguously supports the proposed formulation.

Selective detection of Hg^{2+} ions using HLPy

The designed BODIPY based chemosensor HLPy has 2-amino pyridine as a receptor, where suitable metal ion can coordinate. A systematic metal sensing study was carried out for various biologically important metal ions, such as Co^{2+}, Ca^{2+}, Cd^{2+}, Ag^{+}, Cr^{3+}, Cu^{2+}, K^{+}, Fe^{3+}, Hg^{2+}, Mg^{2+}, Ni^{2+}, Pb^{2+}, Zn^{2+}, Na^{+} and Mn^{2+}, in DMF:H_{2}O (1:9 v/v, 10 mM HEPES buffer, pH 7.4) solution of HLPy. An immediate color change from orange to deep pink was observed only on addition of the Hg^{2+} ion, and no other metal ion showed any significant color change upon addition to the HLPy solution (Fig. S20a). The absorption and emission spectra were recorded for 10 \(\mu\)M solution of HLPy after the addition of 5 \(\mu\)M concentration of different metal ions, resulting in no significant change in the absorbance and emission intensity, except in the case of the Hg^{2+} ion as shown in Figs. 3a and S21. The sequential addition of Hg^{2+} to a solution of HLPy (DMF:H_{2}O, 1:9 v/v, 10 mM HEPES buffer, pH 7.4) resulted in a decrease of absorption bands at 488 nm and appearance of a featured in the red region at 511 nm, suggesting inter-conversion of two species, which we attributed to the free HLPy and BODIPY-Hg^{2+} complex species (Fig. 3b). Similarly, the addition of 5 \(\mu\)M (Hg^{2+}) concentration to the HLPy solution (10 \(\mu\)M) resulting in a “Turn-OFF” fluorescence response under UV irradiation (Fig. S20b). The emission spectral studies suggest up to 94% emission intensity quenching only with Hg^{2+}, indicating HLPy is very selective towards the Hg^{2+} ion (Fig. S22, Supporting Information). Further, the emission titration spectra of HLPy (10 \(\mu\)M) in DMF:H_{2}O (1:9 v/v, 10 mM HEPES buffer, pH 7.4) after the addition of increasing concentrations of Hg^{2+} (0 - 5 \(\mu\)M) were recorded (Fig. S23, Supporting Information). The competitive binding experiments were performed by the addition of excess amounts (10 equivalents) of other metal ions (Fig. 3c). It shows no significant change in the emission spectra induced by Hg^{2+} binding, exhibiting that HLPy can precisely detect mercury ions even in the presence of other metal ions. These findings suggested the likelihood of a complex formation between HLPy and Hg^{2+} ions. Also, the H NMR titration was attained by adding increasing equivalents of Hg^{2+} which shows that the N-H proton signal intensity of HLPy continuously decreases and finally disappeared upon addition of 0.5 equivalent of Hg^{2+} (Fig. S24, Supporting Information). This indicated that Hg^{2+} coordinated to the HLPy after de-protonation of 2-amino pyridine, resulting in \([\text{Hg(LPy)}_2]\), as discussed before. Stern-Volmer constant (\(K_{SV} = 6.37 \times 10^{3} \text{M}^{-1}\)) and binding constant (\(K = 2.04 \times 10^{3} \text{M}^{-1}\)) were determined from the emission titration experiment (Fig. S25a and S25b, Supporting Information). The limit of detection (LOD) was calculated to be 54 nM (Fig. 3d).

Cys detection using \([\text{Hg(LPy)}_2]\) complex

Mercury is known to have a high affinity to the amino acids containing thiol functionality. Therefore, the synthesized complex \([\text{Hg(LPy)}_2]\) was screened with various thiol-containing and other amino acids, such as Cys, Hcy, Met, GSH, Ala, Trp, His and Pro. Initially, we added aqueous solution of biothiols...
and amino acids (20 μM) to [Hg(LPy)2] (10 μM) in DMF:H2O (1:9 v/v, 10 mM HEPES buffer, pH 8.0) solution. No visible color change or fluorescence change was observed except in the case of Cys. It was observed that the addition of Cys to [Hg(LPy)2] produces an immediate color change from deep pink to orange (Inset, Fig. S27, Supporting Information) along with the significant recovery of the fluorescence under UV irradiation (Inset, Fig. 4a). Further, quantitative analysis of Cys detection in the presence of other biothiols and amino acids was carried out over the fluorescence spectrophotometer. None of the biothiols (20 μM) upon addition to [Hg(LPy)2] in DMF:H2O (1:9 v/v, 10 mM HEPES buffer, pH 8.0) showed any significant change in the photophysical properties of the synthesized non-fluorescent [Hg(LPy)2] other than Cys. The addition of aqueous Cys (20 μM) shows significant fluorescence "Turn-ON" feature at 570 nm, which corresponds to the free HLPy (Fig. 4a). The high affinity of the Hg2+ ion towards the thiol group (–SH) of Cys leads to the dissociation of [Hg(LPy)2] into free HLPy and the formation of favored Cys-Hg2+ complex.59 This leads to the reappearance of an orange color fluorescence in the solution under UV irradiation. Similarly, absorption spectra of [Hg(LPy)2] show a blue shift ~11 nm in λmax value i.e. 490 nm (comparable to the free HLPy) upon addition of Cys, resulting an orange colored solution (Fig. S27). The HRMS spectra of [Hg(LPy)2] were recorded after the addition of excess Cys in DMF:H2O, revealing the regeneration of free HLPy m/z = 453.15 (M+H)+ and formation of favored four coordinated Hg(Cys)4 species, m/z = 753.24 (M+DMF), 774.25 (M+Na)+, 792.24 (M+K)+ as presented in Fig. S28 (Supporting Information).

Selectivity and limit of detection of [Hg(LPy)2] towards Cys

Under optimized conditions, the fluorescence spectra of probe [Hg(LPy)2] solution (10 μM) was recorded with an increasing amount (0 - 20 μM) of Cys. The fluorescence intensity of the [Hg(LPy)2] increases with increasing Cys concentration in DMF:H2O (1:9 v/v, 10 mM HEPES buffer, pH 8.0) solution. As shown in Fig. 4b, a linear relationship between the normalized fluorescence intensity of [Hg(LPy)2] and the concentration of Cys was found in the range from 0 to 5 μM. Stern-Volmer constant and association constant between chemosensor [Hg(LPy)2] and Cys come out to be 1.2 × 105 and 1.9 × 1011 M-1, respectively (Figs. S29a and S29b, Supporting Information). The limit of detection (LOD) was calculated to be 29 nM (Fig. 4d), indicating that the designed chemosensor [Hg(LPy)2] was highly sensitive towards Cys. The increase in emission intensity was recorded up to 163.4% (Fig. S30, Supporting Information) upon quantitative addition of 5 μM of Cys to [Hg(LPy)2] solution in DMF:H2O (1:9 v/v, 10 mM HEPES buffer, pH 8.0), resulting in an orange colored solution. This clearly indicates that [Hg(LPy)2] can be used in the colorimetric detection of Cys. Highly selective response to the target analyte is very crucial for biological sample analysis. Therefore, to investigate the selectivity of [Hg(LPy)2] towards Cys, the fluorescence response of [Hg(LPy)2] to a series of interfering species that may coexist with Cys was recorded. The interference presence of species, such as Hcy, Met, GSH, Ala, Trp, His and Pro, with [Hg(LPy)2] showed no change in fluorescence intensity in the absence of Cys. However, when the interference species coexisted with [Hg(LPy)2] and Cys at the same concentration, the fluorescence signals were significantly enhanced (Fig. 4c). These results conclude that [Hg(LPy)2] is very selective and
sensitive specifically for Cys over other similar biothiols, such as Hcy and GSH. This could be because of different reactivity and dissociation patterns of Cys, Hcy and GSH, which have different pKa values (pKa, Cys = 8.0; pKa, Hcy = 8.87; pKa, GSH = 9.20).60-62

Further, the reversibility is an important parameter to evaluate the performance of a chemosensor. Therefore, the emission spectral study was carried out by sequential addition of Cys and Hg2+ to 10 µM solution of [HLPy]2 in DMF:H2O (1:9 v/v, 10 mM HEPEs buffer, pH 8.0), resulting in the “ON-OFF-ON” emission intensity pattern (Fig. S31, Supporting Information). The experiment was repeated up to five cycles without significant change in emission intensity. The reproducibility of fluorescent intensity exhibits the excellent reversibility and reliability of [HLPy]2 as a chemosensor. Also, molecular level arithmetic calculations were performed to demonstrate a simple “NOT-AND” logic gate circuit system with three inputs: IN 1 = [HLPy]2, IN 2 = Cys and IN 3 = Hg2+. The details are given in Supporting Information (Sect. 1.2, Fig. S37).

Effect of pH on photophysical properties of [HLPy]2

In analyte detection, chemosensors are typically affected by the pH of the medium. Hence, the effect of operating pH value on chemosensor sensitivity and detection is extremely significant. The effect of pH in DMF:H2O (1:9 v/v) on the emission intensity of [HLPy]2 was studied in a wide pH range (2.0 – 10.0). The pH of the solution was maintained using different buffer solutions, i.e. CH3COOH/CH3COONa, NaHPO4/Na2HPO4, NH4/NH3•H2O. Within the pH range of 4.0 – 10, the emission response of [HLPy]2 is not significantly affected. However, the emission intensity of [HLPy]2 increases dramatically when the pH is lower than 4.0 (Fig. S32a, Supporting Information). This massive increase in emission intensity might be caused by the structural damage of [HLPy]2 due to the displacement of Hg2+ in the strong acidic condition. Importantly, the emission intensity of [HLPy]2 remains constant during the experiment in the physiological pH range, i.e. 4.0 – 10. Further, experiments to determine the influence of pH on the detection of Cys within the physiological range were also performed. The emission intensity increases on addition of Cys to the solution of [HLPy]2 at all pH values in the range of 4.0 – 10 (Inset Fig. S32a); which shows no interference of pH in the detection of Cys at the physiological pH range. However, the maximum recovery of the emission intensity recorded at 8.0 pH after addition of Cys might be due to the de-protonation of the -SH group in Cys at higher pH, i.e. 8.0 or above (Fig. S32b).25 This infers that 8.0 pH value of the analyte system is ideal for Cys detection. Therefore, all Cys detection studies were carried out at 8.0 pH value.

Performance evaluation of in-situ generated complex species [BODIPY-Hg2+] in Cys detection

The addition of HLPy and Hg(OAc)2 in DMF:H2O (1:9 v/v, 10 mM HEPES buffer) solution results in the formation of in-situ complex species [BODIPY-Hg2+]. To evaluate the sensitivity and selectivity of in-situ generated complex species [BODIPY-Hg2+] in Cys detection, a quantitative amount of biotools was added to the [BODIPY-Hg2+] solution in DMF:H2O (1:9 v/v, 10 mM HEPES buffer, pH 8.0) and the results were compared with the isolated [HLPy]2 complex. As expected, no appreciable changes were observed in the emission intensity of [BODIPY-Hg2+] with any of the amino acids or biotools, i.e. Hcy, Met, GSH, Ala, Trp, His and Pro, except in the case of Cys (Fig. S33, Supporting Information). A colorimetric change from deep pink to orange was observed in the presence of Cys only, along with significant recovery of the fluorescence under UV irradiation (Inset, Fig. S33). A systematic emission intensity titration with an increasing amount of Cys was carried out (Fig. S34a, Supporting Information). It revealed that a much higher concentration of Cys (33 µM) was required to recover complete fluorescence intensity as compared to the isolated complex [HLPy]2 where 20 µM concentration of Cys was used to recover complete fluorescence intensity. Consequently, an inferior detection limit of 146 nM was observed for Cys detection (Fig. S34b). Stern-Volmer constant (Ksv) and binding constant (Kb) values were determined to be 9.85 × 103 and 1.11 × 104 M-1, respectively, which are lower than those observed with the [HLPy]2 complex (Fig. S35, Supporting Information). For in-situ complex, the reversible cycle with Cys could be achieved up to five cycles without much change in the emission intensity (Fig. S36, Supporting Information). The comparative studies reveal that isolated complex [HLPy]2 shows much better performance in terms of quantitative analysis of Cys than in-situ generated complex species [BODIPY-Hg2+]; details are presented in Table S3 (Supporting Information).

Real sample analysis and removal of Hg2+ ion

The sensing and complexation ability of HLPy towards Hg2+ ions prompted us to evaluate the performance of HLPy in detection as well as the removal of Hg2+ from the contaminated water samples. Water samples from various nearby locations were collected and analyzed. For the quantitative measurement, a spiked concentration of Hg2+ was added to the water samples. The satisfactory recovery percent of 97 – 102% shows that the interference from the other compositions of the water samples is negligible on the sensor (Table S4, Supporting Information). Thus, sensor HLPy can be successfully implemented in real environmental sample analysis. Removal of mercury from the contaminated water was performed with a solution of HLPy in DMF:H2O (1:9 v/v). The addition of chemosensor (HLPy) into the contaminated water sample resulted in a colorimetric change from orange to pink. After some time, a deep pink colored precipitation was observed that settled down in the vial, leaving the upper colorless water layer as depicted in Fig. 5. The spectroscopic analysis reveals that the precipitate was nothing but [HLPy]2 complex. Therefore, by simple filtration process, Hg2+ free water can be obtained. Moreover, the removal efficiency of 4 mg of HLPy in 20 mL of contaminated water with Hg2+ ions (0.2 µM) was calculated to be 89 ± 1.2% after 6 h of contact time at ambient temperature (Sect. 1.3, Supporting Information).61
Conclusions

This research work has illustrated synthesis and characterization of a new BODIPY based fluorophore HLPy and its application in Hg²⁺ ion detection. The synthesized HLPy displayed “Turn-OFF” fluorescence response selectively for Hg²⁺ among other metal ions with a limit of detection of 54 nM, and was also used in the removal of Hg²⁺ ions with up to 89% recovery in 6 h from the aqueous solution. Further, two BODIPY based HLPy coordinated to the Hg²⁺ metal center through the deprotonated 2-amino pyridine to yield 2:1 non-fluorescent coordination complex [Hg(LPy)₂]. The non-fluorescent [Hg(LPy)₂] shows excellent “Turn-ON” fluorescent properties specifically for Cys through the de-complexation mechanism with a decent limit of detection (LOD) of 29 nM in a wide physiological pH range, i.e. 4.0 – 10. The reversibility and reusability of the [Hg(LPy)₂] in Cys detection were established up to five cycles by sequential addition of equivalent amounts of Cys and Hg²⁺ without a significant decrease in its performance. The sequential addition of Cys and Hg²⁺ has been used to regulate the “ON-OFF-ON” fluorescence switching of [Hg(LPy)₂], resulting in binary NOT and AND logic gate circuit. The competitive Cys detection analysis of both in-situ BODIPY-Hg²⁺ species and isolated [Hg(LPy)₂] complex reveals that isolated [Hg(LPy)₂] complex has better quantitative performance than in-situ BODIPY-Hg²⁺ species towards Cys detection. Therefore, the synthesized [Hg(LPy)₂] has strong potential as a disposable sensor for selective detection as well as quantification of Cys in biological research and pharmaceutical/food industries. Whereas, the BODIPY based HLPy could be used as a cost-effective, sensitive and selective chemosensor for the detection as well as the removal of Hg²⁺ ion in environmental contaminants.

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Supporting Information

The Supporting Information includes the supplementary FT-IR, absorption, emission, NMR and mass spectra (PDF). This material is available free of charge on the web at http://www.jsac.or.jp/analsci/.

References

1. N. J. Langford and R. E. Ferner, J. Hum. Hypertens., 1999, 13, 651.
2. S.-I. Ohira, Anal. Sci., 2017, 33, 877.
3. Q. Duan, M. Zhang, C. Sheng, C. Liu, L. Wu, Z. Ma, Q. Zhao, Z. Wang, and B. Zhu, Anal. Sci., 2017, 33, 1169.
4. N. Gupta, D. Singhal, A. K. Singh, N. Singh, and U. P. Singh, Spectrochim. Acta, Part A, 2017, 176, 38.
5. D. Teng, K. Mao, W. Ali, G. Xu, G. Huang, N. K. Niazi, X. Feng, and H. Zhang, RSC Adv., 2020, 10, 23221.
6. M. Vedamalai, D. Kedaria, R. Vastia, S. Mori, and I. Gupta, Dalton Trans., 2016, 45, 2700.
7. W. Sun, R. Chen, X. Cheng, and L. Marin, New J. Chem., 2018, 42, 19224.
8. D. Wu, A. C. Sedgwick, T. Gunnlaugsson, E. U. Akkaya, J. Yoon, and T. D. James, Chem. Soc. Rev., 2017, 46, 7105.
9. K. R. Kim, H. J. Kim, and J. I. Hong, Anal. Chem., 2019, 91, 1353.
10. V. Kumar, P. Kumar, S. Kumar, D. Singhal, and R. Gupta, Inorg. Chem., 2019, 58, 10366.
11. S. Mondal, S. K. Manna, S. Pathak, A. Ghosh, P. Datta, D. Mandal, and S. Mukhopadhyay, New J. Chem., 2020, 44, 7954.
12. A. Loudet and K. Bugess, Chem. Rev., 2007, 107, 4891.
13. S. Madhui, R. Kalyuvarasi, S. K. Basu, S. Jadhav, and M. Ravikanth, J. Mater. Chem. C, 2014, 2, 2534.
14. P. Kaur and K. Singh, J. Mater. Chem. C, 2019, 7, 11361.
15. N. Boens, V. Leen, and W. Dehaen, Chem. Rev., 2012, 11, 1130.
16. D. W. Domaille, L. Zeng, and C. J. Chang, J. Am. Chem. Soc., 2010, 132, 1194.
17. S. C. Dodani, S. C. Leary, P. A. Cobine, D. R. Wing, and C. J. Chang, J. Am. Chem. Soc., 2011, 133, 8606.
18. R. Ziessel and A. Harriman, Chem. Commun., 2011, 47, 611.
19. G. Ulrich, R. Ziessel, and A. Harriman, Angew. Chem. Int. Ed., 2008, 47, 1184.
20. E. Weerapana, C. Wang, G. M. Simon, F. Richter, S. Khare, M. B. D. Dillon, D. A. Bachovchin, K. Mowen, D. Baker, and B. F. Cravatt, Nature, 2010, 468, 790.
21. L. B. Poole, Free Radical Biol. Med., 2015, 80, 148.
22. S. Lavoie, M. M. Murray, P. Deppen, M. G. Knyazeva, M. Berk, O. Boulat, P. Bovet, A. I. Bush, P. Conus, D. Copolov, E. Fornari, R. Meuli, A. Solida, P. Vinian, M. Cuenod, T. Buclin, and K. Q. Do, Neuropsychopharmacology, 2008, 33, 2187.
23. K. G. Reddie and K. S. Carroll, Curr. Opin. Chem. Biol., 2008, 12, 746.
24. N. C. Plaza, M. R. García-Galbis, and R. M. Martínez-Espinosa, Molecules, 2018, 23, 575.
25. X. He, X. Wu, W. Shi, and H. Ma, Chem. Commun., 2016, 52, 9410.
26. Z.-H. Fu, X. Han, Y. Shao, J. Fang, Z.-H. Zhang, Y.-W. Wang, and Y. Peng, Anal. Chem., 2017, 89, 1937.
27. D. Chen, Z. Long, Y. Dang, and L. Chen, Analyst, 2018, 143, 5779.
28. D. H. Ma, D. Kim, T. Akisawa, K.-H. Lee, K.-T. Kim, and W. Kozubski, Neuropsychopharmacology, 2016, 41, 952.
29. I. S. Ahmed, J. Selhub, P. F. Jacques, I. H. Rosenberg, R. B. D’Agostino, P. W. F. Wilson, and P. A. Wolf, Nature, 2002, 416, 476.
30. X. Chen, Y. Zhou, X. Peng, and J. Yoon, Chem. Soc. Rev., 2010, 39, 2120.
31. Y. Zhou and J. Yoon, Chem. Soc. Rev., 2012, 41, 52.
32. L. Y. Niu, Y. Z. Chen, H. R. Zheng, L. Z. Wu, C. H. Tung, and Q. Z. Yang, Chem. Soc. Rev., 2015, 44, 6143.
33. J. Shaoa, H. Guoa, S. Ji, and J. Zhaoa, Biosens. Bioelectron., 2011, 26, 3012.
34. D. H. Ma, D. Kim, T. Akisawa, K.-H. Lee, K.-T. Kim, and K. H. Ahn, Chem. Asian J., 2014, 9, 894.
35. D. H. Ma, D. Kim, E. Seo, S. J. Lee, and K. H. Ahn, Analyst, 2015, 140, 422.
36. Q. Wu, Ji. Zhou, Y. Wu, C. Yu, E. Hao, and L. Jiao, New J. Chem., 2016, 40, 1387.
39. N. Wang, Y. Wang, J. Gao, X. Ji, J. He, J. Zhang, and W. Zhao, *Analyst*, 2018, 143, 5728.
40. N. Wang, X. Ji, H. Wang, X. Wang, Y. Tao, W. Zhao, and J. Zhang, *Anal. Sci.*, 2020, 36, 1317.
41. Q. Li, Y. Guo, and S. Shao, *Sens. Actuators B*, 2012, 171–172, 872.
42. N. Kaur, P. Kaur, and K. Singh, *RSC Adv.*, 2014, 4, 29340.
43. O. G. Tsay, K. M. Lee, and D. G. Churchill, *New J. Chem.*, 2012, 36, 1949.
44. C.-C. Zhao, Y. Chen, H.-Y. Zhang, B.-J. Zhou, X.-J. Lu, and W.-F. Fu, *J. Photochem. Photobiol., A*, 2014, 282, 41.
45. Y. Wang, Q. Meng, Q. Han, G. He, Y. Hu, H. Feng, H. Jia, R. Zhang, and Z. Zhang, *New J. Chem.*, 2018, 42, 15839.
46. M. D. Gholami, S. Manzhos, P. Sonar, G. A. Ayoko, and E. L. Izake, *Analyst*, 2019, 144, 4908.
47. D. B. G. Williams and M. Lawton, *J. Org. Chem.*, 2010, 75, 8351.
48. M. Kumar, N. Gupta, and A. P. Singh, *Anal. Sci.*, 2020, 36, 659.
49. SMART: Bruker Molecular Analysis Research Tool, Ver. 5.618, Bruker Analytical X-ray System, 2000.
50. SAINT-NT, Ver. 6.04, Bruker Analytical X-ray System, 2001.
51. SHELXTL-NT, Ver. 6.10, Bruker Analytical X-ray System, 2000.
52. J. H. Hildebrand and H. A. Benesi, *J. Am. Chem. Soc.*, 1949, 71, 2703.
53. A. Shrivastava and V. B. Gupta, *Chron. Young Sci.*, 2011, 2, 21.
54. H. Boaz and G. K. Rollefson, *J. Am. Chem. Soc.*, 1950, 72, 3435.
55. Y. Zhang, C. Zhang, Y. Wu, B. Zhao, L. Wang, and B. Song, *RSC Adv.*, 2019, 9, 23382.
56. S. Madhu, D. K. Sharma, S. K. Basu, S. Jadhav, A. Chowdhury, and M. Ravikanth, *Inorg. Chem.*, 2013, 52, 11136.
57. J. S. Renny, L. L. Tomasevich, E. H. Tallmadge, and D. B. Collum, *Angew. Chem. Int. Ed.*, 2013, 52, 2.
58. K. Nakamoto, "Infrared and Raman Spectra of Inorganic and Coordination Compounds", 1986, John Wiley & Sons, New York.
59. F. Jalilehvand, B. O. Leung, M. Izadifard, and E. Damian, *Inorg. Chem.*, 2006, 45, 66.
60. Y. Yang, F. J. Huo, C. Yin, J. Chao, and Y. Zhang, *Dyes Pigm.*, 2015, 114, 105.
61. R. E. Benesch and R. Benesch, *J. Am. Chem. Soc.*, 1955, 77, 5877.
62. Y. Yue, F. Huo, X. Li, Y. Wen, T. Yi, J. Salamanca, J. O. Escobedo, R. M. Strongin, and C. Yin, *Org. Lett.*, 2017, 19, 82.
63. A. Mondal, A. R. Chowdhury, S. Bhuyan, S. K. Mukhopadhyaye, and P. Banerjee, *Dalton Trans.*, 2019, 48, 4375.