A New Thiophene-Appended Fluorescein-Hydrazone-Based Chromo-Fluorogenic Sensor for the Screening of Hg²⁺ Ions in Real Water Samples

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ABSTRACT

A simple yet efficient, Hg²⁺ selective sensor, based on a fluorescein-thiophene conjugate, namely, (Z)-3',6'-dihydroxy-2-(((3-methylthiophen-2-yl)methylene)amino)spiro[isoindoline-1,9'-xanthen]-3-one (FT) has been designed and synthesized. The compound was well characterized by analytical techniques such as HR–MS, FTIR, UV–visible absorption, steady-state fluorescence and time-resolved spectrophotometric techniques. Comprehensive analysis demonstrated FT as a highly specific and ultra-sensitive sensor towards Hg²⁺ ions in presence of several other interfering metal ions in ethanol/H₂O (9:1, v/v) at pH = 7.2 buffered with 10 mM HEPES buffer at room temperature. The complexation of the probe FT with Hg²⁺ was well confirmed by ESI–MS and FTIR spectral analysis. The detection limit of the probe FT towards Hg²⁺ was achieved down to 137 nM. The structure of ligand FT and FT-Hg²⁺ complex were well corroborated by theoretical studies as well. In addition, the FT-Hg²⁺ complex was found to be reversible in presence of di-sodium EDTA and hence decipher its recyclability capabilities. The significance of the present probe FT lies in its successful application for the detection and quantification of Hg²⁺ in real water samples and logic gate fabrication for future incorporation in small organic molecule based efficient molecular devices.
1. Introduction

A rising upsurge in the quantitative screening of non-essential heavy metals is witnessed in recent times due to their grievous detrimental consequences in normal human physiological functioning and global ecosystem.\textsuperscript{[1]} As one of the systemic toxicants, mercury rank at the top of priority metals for the ability to induce multiple organ failures, even at nanomolar level of exposure.\textsuperscript{2} Its widespread distribution in the environment due to its potential reported applications in agriculture, industry, domestic, medical and technological domains, has instigated an urgent need of highly facile and novel analytical methodology to monitor trace levels of this analyte of high concern in different industrial, environmental and biological samples.\textsuperscript{[3-5]}

The omnipresent three chemical forms of mercury, i.e., elemental mercury (Hg\textsuperscript{0}) and two cationic forms (Hg\textsuperscript{2+} and Hg\textsuperscript{2+}) in the environment, enters into the food chain and affect global ecological cycle. Natural inclusion of mercury in water sources due to off-gassing from earth’s crust, is readily transformed to organic methyl mercury (CH\textsubscript{3}Hg) by marine microorganisms, which enters into fishes and eventually those contaminated fishes are ingested by human.\textsuperscript{[6,7]} Due to high lypophilicity and ability to cross blood-brain-barrier (BBB), mercury gets easily absorbed and accumulated in liver, kidney and neurological tissues.\textsuperscript{[8,9]} Prolonged accumulation of these species lead to oxidative stress, increased production of intracellular reactive oxygen species (ROS), DNA damage, genotoxic alterations, severe neurotoxicity, nephrotoxicity and gastrointestinal toxicity.\textsuperscript{[10-14]} Due to severity of mercury toxicity, the maximum permissible limit of mercuric ions in drinking water was recommended as 2 μg/L (10 nM) by the United States Environmental Protection Agency (US EPA).\textsuperscript{[15]}

Among diverse potent analytical methodologies, chromo-fluorogenic chemosensing approach has witnessed an exponential growth as a ‘star’ technique that are featured with unique advantages of high specificity, excellent sensitivity, easy-to-design, lower detection limits, low cost, good biocompatibility and rapid on-site detection capability, among a few.\textsuperscript{[16-18]} The main structural features of chromo-fluorogenic sensor are composed of signalling unit (such as highly efficient fluorophores, chromophores, nano-aggregates, etc.) and recognition unit (moiety consisting of suitably arranged multiple donors to bind with target analyte).\textsuperscript{[19]} The mechanism is based on host-guest interaction between recognition unit and target analyte that triggers energy/electron/charge transfer that may operate within sensor (such as photo-induced electron transfer (PET), intramolecular charge transfer (ICT), fluorescence resonance energy transfer (FRET), etc.) or may extend to analyte (such as ligand-to-metal charge transfer (LMCT), heavy atom effect, etc.) that altogether changes the spatial electronic distributions and subsequent photophysical properties of the fluorophores producing distinct optical signal as output response.\textsuperscript{[20]}

Among diverse sensory systems, small-molecule based fluorescent probes are highly effective and well-suited for rapid on-site and in vivo monitoring of Hg\textsuperscript{2+} in different real time environmental and biological samples, respectively.\textsuperscript{[21-23]} However, the less effective ‘turn-off’ fluorescence detection mechanism is predominantly observed due to many factors such as using mixed aqueous medium that promotes aggregation caused quenching effect of traditional fluorophores with planar π-conjugated systems or heavy atom effect leading to
emission quenching effect.\textsuperscript{[24,25]} In this context, the development of highly efficient small-molecule based ‘turn-on’ fluorescence detection system, featured with high signal contrast, enhanced sensitivity and reduction in false positive signals, is highly beneficial and challenging by surpassing the above limitations.\textsuperscript{[26-28]}

Fluorescein, as an excellent chromophore, featured with high fluorescence quantum yield, large molar extinction co-efficient, good water solubility, easy synthetic routes and high photostability, has been paid much less attention as fluorescent sensors in comparison with other fluorophores such as rhodamine, coumarin, pyrene, etc, thus creating enough room to develop highly sensitive, rapid, cost-effective and portable sensory probes that are selective to target Hg$^{2+}$ ions.\textsuperscript{[29-31]} The unique characteristic feature of fluorescein lies in its two distinctive structures, spirocyclic and open-formed structures with distinct spectroscopic properties. The non-fluorescent and colorless spirocyclic form of fluorescein derivatives can be converted to highly conjugated open form which is triggered by interaction with specific analyte resulting in the appearance of solution color change and brilliant fluorescent properties.\textsuperscript{[32-35]} Inspired by this activity based sensing approach, we have endeavoured to design and synthesize thiophene appended fluorescein-hydrazone derivative and analyze its potential as real-time sensory system for Hg$^{2+}$ ions in aqueous medium through different experimental and theoretical techniques and contribute to the family of versatile sensors for this hazardous analyte detection process.

2. Result and Discussion

2.1. Synthesis and characterization

Schiff base (Z)-3',6'-dihydroxy-2-(((3-methylthiophen-2-yl)methylene)amino)spiro[isoindoline-1,9'-xanthen]-3-one (FT) could be facieely prepared by simple one-step condensation reaction between equimolar amount of fluorescein hydrazone (FH) and 3-methylthiophene-2-carboxaldehyde as shown in Scheme 1 and subsequently characterized by single crystal X-ray structural analysis, FT-IR, HR-MS and elemental analysis.\textsuperscript{[36]} The details of synthetic procedure is given in supplementary information. The X-ray crystallographic structure indicates that O31, N23 atoms in FH moiety and S25 atom of 3-methylthiophene-2-carboxaldehyde moiety lies in almost same plane and can provide suitable coordination sites for binding with a certain metal ion (Figure 1). All the crystal parameters are depicted in Table S1.
Scheme 1. Synthetic route for 3-methylthiophene-2-carboxaldehyde fluorescein hydrazone (FT).
2.2. Selection of solvent system

The selection of appropriate solvent medium for investigating sensing performance of FT was first carried out through UV-visible and fluorescence spectroscopy. As shown in Figures S1 and S2 in the supplementary file, the absorption and fluorescence spectra of FT (20 μM) in ethanol are featured with highly well-defined, structured absorption bands and emission band with appreciable intensities among all other solvent system tested where feeble emission bands are observed with less structural features to be defined accurately. So, ethanol was chosen as the solvent of choice for the next phase of experiments.

Next, the absorption and fluorescence changes of FT were monitored with increase in water content where ethanol was used as the co-solvent. As shown in Figures S3 and S4, FT displays maximum absorbance of the absorption band centered at 220 nm and highest fluorescence intensity of emission band centered at 305 nm in EtOH/H₂O (9:1, v/v) solvent system respectively. Thus, the as synthesized probe FT exhibits the superior structural features as evident from above experiment and thus the best proportion of EtOH/H₂O (9:1, v/v) solvent mixture can be chosen for performing sensing of mercuric ions.

Further, we performed spectrofluorometric titrations of FT by adding different metal ions in different semi-aqueous media (Figure S5). Interestingly, we found a significant fluorescence enhancement (88 fold) at 514 nm when excited at 340 nm only in presence of Hg²⁺ in EtOH/H₂O (9:1, v/v; pH=7.0).
2.3. UV-visible spectroscopic analysis of interaction of FT with Hg\(^{2+}\)

2.3.1. UV-visible absorption titration study

The UV-visible absorption spectrum of free probe FT (20 µM) in EtOH/H\(_2\)O (9:1, v/v; pH=7.2 with 10 mM HEPES buffer) displays two strong absorption bands centered at 276 nm and 342 nm, which are the characteristics of thiophenyl and closed spirolactum form of fluorescein moieties respectively.\(^{37,38}\) The interaction of Hg\(^{2+}\) (0-325 µM) with FT was analyzed by performing UV-visible spectrophotometric titration in EtOH/H\(_2\)O (9:1, v/v) buffered at pH=7.2. As shown in Figure 2 A, upon gradual addition of Hg\(^{2+}\) to the solution of FT (20 µM), the initial characteristic of absorption bands of free probe centered at 276 nm and 342 nm gradually decreased in intensity, while a new broad absorption band at 456 nm with a shoulder band at 486 nm (which represent characteristic absorption of fluorescein) was concomitantly appeared and its absorption intensity was increased with increasing the concentration of Hg\(^{2+}\) which became saturated at about 325 µM of Hg\(^{2+}\) ions. Correspondingly, the UV-visible recognition of Hg\(^{2+}\) was readily realized by observing a clear change of solution color from colorless to deep yellowish brown. The noticeable spectral and color changes clearly signifies Hg\(^{2+}\) induces ring opening reaction of spirocycle from of FT and formation of stable FT-Hg complex as evident from clear isosbestic point at 384 nm. Thus the probe FT can suitably be utilized as a sensitive colorimetric sensor for Hg\(^{2+}\) ions in semi-aqueous medium. The absorbance changes at 342 nm and 456 nm were plotted with the variation of Hg\(^{2+}\) concentrations which shows almost linear intensity changes in wide concentration range, indicating feasibility of the sensor to be applied colorimetrically for monitoring of real samples with wide concentration variations of the concerned analyte (Figure 2 B).

**Figure 2** A. UV-visible absorption changes of FT (20 µM) upon addition of increasing concentration of Hg\(^{2+}\) ions in EtOH/H\(_2\)O (9:1, v/v) at pH = 7.2 buffered with 10 mM HEPES buffer at room temperature ([Hg\(^{2+}\)] = 0 – 325µM); B. Absorbance changes of FT at 342 nm and 456 nm with varying Hg\(^{2+}\) concentration.

2.3.2. Selective response of FT towards Hg\(^{2+}\) over other metal ions
In addition to sensitive response, the selective binding affinity of free probe FT towards various other metal ions in EtOH/H₂O (9:1, v/v) at pH = 7.2 buffered with 10 mM HEPES buffer at room temperature, were investigated by absorption spectral changes as depicted in Figure 3A. It could be observed that except Hg²⁺, the addition of 20 equivalents excess of various metal ions such as Al³⁺, Ba²⁺, Ca²⁺, Cd²⁺, Co²⁺, Cr³⁺, Fe³⁺, Li⁺, Mg²⁺, Mn²⁺, Na⁺, Pb²⁺, Ni²⁺, Zn²⁺ did not induce any effect on the UV-visible spectrum of FT and the solution color remained almost unchanged (colorless). However, upon addition of 20 equivalents of Hg²⁺, the solution color instantaneously changed from colorless to brownish yellow accompanied with the appearance of a new broad absorption band in the visible region 400-500 nm (λ_max = 450 nm) which clearly emphasized intermolecular charge transfer interaction between Hg²⁺ and FT promoting conformational transformation from spirocyclic to ring opened oxide form of fluorophore. In contrast to Hg²⁺ ions, other metal ions could not be able to perturb the ligand solution color as depicted by the photographic images both under daylight and UV light irradiation (Figures S6 and S7). The spectral perturbation effect of FT in response to various metal ions were also represented in the form of bar diagram (Figure 3B) which clearly imply super selective response attribute of FT in presence of Hg²⁺ only.

Figure 3A. UV-visible absorption spectra of FT (20 µM) upon addition of 20 equivalents of various metal ions in mixed solution of EtOH/H₂O (9:1, v/v) at pH = 7.2 buffered with 10 mM HEPES buffer at room temperature. B. Corresponding bar diagram.

2.4. Fluorescence recognition of Hg²⁺

2.4.1. Fluorescence titration study

Fluorescence titration experiment was carried out to have a better insight into the emission properties of free sensor and photophysics lying behind selective binding interaction between FT and Hg²⁺ ions. The free chemosensor FT (20 µM) in EtOH/H₂O (9:1, v/v; at pH = 7.2, 10 mM HEPES buffer) reaction medium, displayed moderately fluorescent emission band centered at 425 nm (λ_ex = 340 nm) with a quantum yield (φ) of 0.0864. Further, no characteristic emission band above 500 nm indicated the ring closed five membered spirolactum structure of fluorescein core (Figure 4A).
However, upon gradual 5 μM incremental addition of aliquots of Hg$^{2+}$ ions in water (0-160 μM), a vivid enhancement of fluorescence intensity at 512 nm resulting in a 51 fold increase in emission intensity (φ = 0.7714) and a slight decrement of emission intensity at 430 nm upon excitation at 340 nm was perceived. The occurrence of sharp iso-emissive point at 475 nm implies the spirocyclic-ring opening phenomena of fluorescein fluorophore. The ratio of fluorescence intensities at 512 nm and 430 nm ($I_{512}/I_{430}$) plotted against varying concentration of Hg$^{2+}$ ions also implies a linear trajectory of intensity change over a wide concentration range making the probe feasible enough to monitor wide concentrations of Hg$^{2+}$ ions in practical samples (Figure 4 B).

The mechanism of sensing Hg$^{2+}$ ions by probe FT can be explained as follows. The ratiometric fluorescence emission at two different emission wavelengths implies a change in emissive unit between free probe and complexed species. Complexation of Hg$^{2+}$ ions with the chelating probe resulted in the formation of rigid host-guest molecular assembly which can effectively suppress non-radiative deactivation channels of the photoexcited fluorophores through different quenching processes such as photo-induced electron transfer (PET), inhibition of C=N isomerization and rotation across single bonds, leading to efficient chelation enhanced fluorescence effect (CHEF).\[39] A slight spectral overlap between thiophenyl emission with the absorption of fluorescein hydrazone - Hg$^{2+}$ complex also encourages the two moieties to act as a donor-acceptor pair which results in energy transfer from thiophene donor to fluorophore acceptor resulting in opening of spirocyclic ring leading to FRET process (Figure S8).\[40,41] Thus, a synergistic effect of CHEF and FRET might be the predominant photophysical processes which direct the selective fluorogenic signaling mechanism of FT with Hg$^{2+}$ ions.

2.5. Detection limit : Sensitivity of the probe

![Figure 4 A. Spectrofluorophotometric titration of FT (20 μM) in HEPES buffered EtOH/H$_2$O (10 mM, 9:1, v/v, pH = 7.2) by the incremental addition of Hg$^{2+}$ with $\lambda_{ex} = 340$ nm, $\lambda_{em} = 430$ nm, $\lambda_{em} = 512$ nm, slit = 5.0 nm. Inset: Fluorescence photographs of FT in absence and presence of Hg$^{2+}$ ions under UV light irradiation. B. The fluorescence intensity ratio of FT ($I_{512}/I_{430}$) with varying concentration of Hg$^{2+}$.

Figure 4 B]
The detection limit of FT for Hg\(^{2+}\) was determined by \(3\sigma/m\) method, where, ‘\(\sigma\)’ is the standard deviation of blank measurements which is obtained by measuring fluorescence emission at emission peak position of 5 different concentrations of FT solution and ‘\(m\)’ is the slope of the calibration curve obtained by plotting fluorescence intensity with Hg\(^{2+}\) concentrations within the linear concentration range.

From fluorescence titration data, a plot of fluorescence intensity changes at 510.5nm with respect to Hg\(^{2+}\) concentration shows a linear concentration range 60-120 \(\mu\text{M}\) Hg\(^{2+}\) ions (Figure 5 A). A linear fit plot within this concentration range gives the value of slope \(m = 92.075\) (\(R^2 = 0.9997\)) (Figure 5 B). Thus, the limit of detection (LOD) value is found to be 137 nM signifying its alluring sensitivity response.

![Figure 5](image)

**Figure 5** A. Plot of fluorescence intensity changes of FT with varying Hg\(^{2+}\) at 510.5 nm. B. A linear fit plot for calculation of LOD parameter of FT.

### 2.6. Binding stoichiometry and affinity

In order to have an in depth knowledge of binding affinity and stoichiometry on binding between FT and Hg\(^{2+}\), Benesi-Hildebrand equation and Job’s plot analysis was used respectively.\(^{[42]}\) Job’s plot analysis was performed with \([\text{FT}] + [\text{Hg}^{2+}] = 50 \mu\text{mol/L}\) of total concentration. The absorbance changes at 534 nm were plotted as a function of metal mole fraction (\(X_M\)). The absorbance maximum was obtained at a mole fraction of 0.502 which implies 1:1 complexation between FT and Hg\(^{2+}\) (Figure 6).

According to Benesi-Hildebrand expression, the measured fluorescence \([F_{\text{max}} - F_0]/F_x - F_0\) at 510.5 nm where \(F_0\), \(F_x\) and \(F_{\text{max}}\) are the fluorescence of the sensor in absence of Hg\(^{2+}\), at a certain concentration of Hg\(^{2+}\) and at the concentration of saturation point respectively, varied as a function of \(1/[\text{Hg}^{2+}]\) in a good linear relationship (\(R^2 = 0.9948\)) (Figure 7) which implies 1:1 stoichiometry between FT and Hg\(^{2+}\). This 1:1 binding stoichiometry of the complex is also ascertained from HR-MS and FT-IR spectra of the formed complex FTM (Figures S9-S11). The association constant of FT with Hg\(^{2+}\) was calculated from slope which is estimated to be \(5.076 \times 10^3 \text{ M}^{-1}\) indicating a stable complexation between probe and the concerned analyte.
Figure 6. Job’s plot of FT with Hg$^{2+}$ in aqueous solution. Total concentration of [FT]+[Hg$^{2+}$] was kept constant at 50 µmol/L. The absorbance at 534 nm was used.

Figure 7. The measured intensity $[F_{\text{max}} - F_0] / [F_x - F_0]$ at 510.5 nm varied as a function of $1/[\text{Hg}^{2+}]$ ($10^6$) in a linear relationship ($R^2 = 0.9948$). FT (20 µmol L$^{-1}$) was treated with various concentrations of Hg$^{2+}$ (0 – 160 µmol L$^{-1}$).
2.7. Single and dual metal competition studies

The newly synthesized fluorogenic sensor must have sensitive performance towards one particular analyte over other potentially competing analytes for its utilization in quantitative monitoring of practical samples. Therefore, the fluorescence response of FT (20 µM) in the presence of various metal ions (5 equivalents) were investigated. As depicted in Figure 8, addition of metal ions such as Al$^{3+}$, Ba$^{2+}$, Ca$^{2+}$, Cd$^{2+}$, Co$^{2+}$, Cr$^{3+}$, Fe$^{3+}$, Li$^+$, Mg$^{2+}$, Mn$^{2+}$, Na$^+$, Pb$^{2+}$, Ni$^{3+}$, Zn$^{2+}$ (100 µM) in FT (20 µM) solution resulted a negligible fluorescence intensity changes but addition of Hg$^{2+}$ ions (5 equivalents) resulted a vivid (74 fold) fluorescence emission enhancement at 512 nm upon excitation at 340 nm.

![Figure 8](image)

**Figure 8.** Fluorescence spectra of FT (20 µM) in presence of various of metal ions, such as Al$^{3+}$, Ba$^{2+}$, Ca$^{2+}$, Cd$^{2+}$, Co$^{2+}$, Cr$^{3+}$, Fe$^{3+}$, Li$^+$, Mg$^{2+}$, Mn$^{2+}$, Na$^+$, Pb$^{2+}$, Ni$^{3+}$, Zn$^{2+}$ (100 µM). The excitation was at 340 nm, emission was at 512 nm, excitation/emission slit = 5/5 nm.

Furthermore, dual metal competitive analysis was performed to investigate interference influences, if any, presented by the presence of other potentially competitive cations in the presence of Hg$^{2+}$ ions. So, the fluorescence emission of FT (20 µM) in ethanol/H$_2$O buffer solution (9:1, v/v, pH = 7.2) in the presence of equimolar amounts of Hg$^{2+}$ and other metal ions (100 µM + 100 µM) were recorded and presented as bar diagram in Figure 9. Except for Cu$^{2+}$ and Pb$^{2+}$, which resulted a little quenching of fluorescence emission of FT-Hg$^{2+}$ complex due to paramagnetic nature of Cu$^{2+}$ and heavy metal quenching effect of Pb$^{2+}$, other
competitive metal ions did not reveal any noticeable interference in the detection of Hg\(^{2+}\) ions. These facts clearly reveal that FT can serve as a highly selective “turn on” fluorogenic chemosensor for Hg\(^{2+}\) in aqueous buffer solution potentially active for on-site quantitative analysis of trace Hg\(^{2+}\) ions.

![Figure 9](image_url)

**Figure 9.** Single and dual metal ions competitive fluorescence response of FT in ethanol/H\(_2\)O (9:1; v/v, 10 mM HEPES buffer, pH = 7.2) solution. Excitation = 340 nm, emission = 512 nm, slit = 5/5 nm.

### 2.8. pH dependence studies

As fluorescence properties of fluorescein is highly pH dependent due to its existence in cationic, neutral, mono-anionic and di-anionic forms under different pH conditions. So, for its practical utilization in biological samples, it must exhibit sensitive response within a wide range of pH. Thus the optimization of pH is necessary for developing efficient sensor.\(^{[43]}\) As depicted in **Figure 10** A, the emission intensities of metal free sensor FT was too weak within a wide range of pH from 2-12 with a slight inflation within pH 6-8. Such weak and unchanged emission suggests the spirocyclic form of fluorescein part of the sensor FT and this closed form (tautomer) was unresponsive towards change of pH conditions. However, upon addition of 125 µM of Hg\(^{2+}\), the fluorescence intensity at 512 nm, upon excitation at 340 nm, was significantly amplified within pH range from 6.0-8.0, which implies Hg\(^{2+}\)-induced spiro-ring opening and subsequent formation of efficient FT-Hg ensemble featured with more rigid molecular structure by suppressing intramolecular rotations and C=N...
isomerization leading to pronounced CHEF effect. Again at very low pH 2.0-3.0, fluorescence intensities were too feeble, that might be due to breakage of imine linkage of FT, which prevents coordination of Hg$^{2+}$, while at very high pH 11.0-12.0, de-protonation of FT followed by spirocycle ring opening and further coordination with Hg$^{2+}$ to form FT-Hg complex takes place in highly alkaline solution.$^{[44]}$ The fluorescence intensity changes of FT at 512 nm were represented as histogram plots with respect to pH change from 2-12 (Figure 10 B). Thus, in the present study, by considering fluorescence signals and stability of complex, pH 6-9 can be considered to be the most optimized and effective pH range to monitor Hg$^{2+}$ ions. In our present work, pH 7.2 with 10 mM HEPES buffer solution was chosen for carrying out all the experiments throughout the paper for monitoring Hg$^{2+}$ ions.

![Figure 10 A. Fluorescence intensity versus pH plot at 512 nm of free FT (20 µM) in ethanol-H$_2$O (9:1, v/v) (denoted by cyan circle) and in the presence of Hg$^{2+}$ (125 µM) (denoted by green circles). B. Fluorescence intensity (at 512 nm) vs pH plot (histogram plot).](image)

### 2.9. Reversibility studies

A novel chemosensor must be featured with reversible binding characteristics for its efficient multiple sensing applications. In order to check whether the proposed complex could be reversed back to bare state or not, reversibility experiment was performed. The addition of excess EDTA (10 equivalents) to the FT (20 µM) solution with Hg$^{2+}$ (100 µM) resulted in gradual quenching of fluorescence intensity at 512 nm and the initial spectral characteristics of bare probe reverted back (Figure 11 A). Further, second time addition of Hg$^{2+}$ (5 equivalents) to the same solution resulted in sharp fluorescence enhancement as observed in first cycle and this alternate addition of Hg$^{2+}$ and EDTA stimuli was continued upto 5 cycles (Figure 11 B). This quenching of fluorescence can be attributed to the fact that Na$_2$EDTA has stronger affinity for Hg$^{2+}$ and the association constant of binding of EDTA with Hg$^{2+}$ might be higher than that of FT-Hg complex resulting in demetallation of Hg$^{2+}$ from the probe-Hg complex with subsequent releasing and closing of spirolactum ring of free fluorophore. These results demonstrate that the sensor can be regenerated and reused upto 5 cycles retaining same level of efficiency and provides opportunity for device fabrication for practical sensory application.
**Figure 11** A. Reversibility of Hg$^{2+}$ coordination to FT by Na$_2$EDTA. Initial [FT] = 20 µmol/L in ethanol/water (9:1, v/v, pH = 7.2); alternate addition of Hg$^{2+}$ (5 equivalents) and Na$_2$EDTA (10 equivalents); B. Plot of fluorescence intensity (512 nm) versus number of cycle for reversible addition of Hg$^{2+}$ (5 equivalents) and Na$_2$EDTA (10 equivalents) to FT solution (20 µmol/L) upto 5 cycles. Fluorescence intensity was monitored at 512 nm. Excitation = 340 nm; Emission = 512 nm; slt = 5.0 nm.

### 2.10. Sensor response time

Time dependence of fluorescence spectral response of FT (20 µM) towards Hg$^{2+}$ (140 µM) in ethanol/water buffer solution (9:1, v/v, pH = 7.2) was investigated by excitation at 340 nm and monitoring fluorescence intensity at 512 nm at an equal span of 10 seconds (**Figure 12** A). It can be observed that the system get to maximum within 200 seconds and remained almost unchanged with more reaction time under identical conditions. Such leveling off spectral response indicated saturation of spirocyclic ring opening assisted complexation reaction. This signify that the sensor could be used for real time monitoring of Hg$^{2+}$ and 5 minutes was choosen as the complete recognition time in all the experiments throughout this paper. The relative fluorescence intensity (I/I$_0$) at 512 nm was also plotted as a function of time (0 – 240 seconds) to visualize the changes clearly (**Figure 12** B).
Figure 12 A. Fluorescence spectral responses of FT (20 µmol/L) towards Hg²⁺ (140 µmol/L) in aqueous buffer solution at an equal interval of 10 seconds. B. The relative fluorescence intensity (I-I₀/I₀) at 512 nm as a function of time (0 – 240 seconds).

2.11. Time-resolved photoluminescence decay analysis (TRPL)

The time-resolved photoluminescence measurements were performed to have a quantitative picture of the mechanism of turn-on sensor responses of FT towards Hg²⁺ ions (Figure 13). The measurement of fluorescence lifetime is more robust than the measurement of steady state fluorescence intensity as it is independent of excitation intensity as well as concentration of the fluorophores. The fluorescence lifetime of FT in ethanol-water (9:1; v/v) exhibited bi-exponential decay behavior with lifetimes of 0.72 ns (89%) and 7.98 ns (11%) in the absence of Hg²⁺ ions. Interestingly, a large change is revealed upon addition of Hg²⁺ to the FT solution. Longer fluorescence lifetimes of 2.15 ns (5%) and 4.05 ns (95%), corresponding to short-lived and long-lived decay components respectively, were observed. The average fluorescence lifetime (τavg) increased from 1.53 ns to 3.95 ns upon addition of Hg²⁺ to FT solution. All the photo-physical parameters are enlisted in Table 1. The radiative rate constant (k_r) and non-radiative rate constant (k_nr) are calculated from average fluorescence lifetime and quantum yield to gain insight into the sensing mechanism. The weak fluorescence of FT can be attributed due to prominent non-radiative decay of excited state which might be due to efficient intra-molecular photo-induced electron migration from nitrogen atom of imine to fluorescein moiety which is supported by larger values of non-radiative rate constant (k_nr = 0.595 x 10⁹ sec⁻¹) and much lower value of radiative rate constant (k_r = 0.0563 x 10⁹ sec⁻¹). The co-ordination of FT with Hg²⁺ induced structural rigidity, by inhibiting the free rotations of resultant complex and PET is efficiently suppressed due to involvement of nitrogen atom of imine in coordination to Hg²⁺ ions leading to CHEF effect. This blockage of non-radiative deactivation channel in resultant ring opened complex is well evidenced with the much lower value of non-radiative rate constant (k_nr = 0.597 x 10⁹ sec⁻¹) and increased value of radiative rate constant (k_r = 0.195 x 10⁹ sec⁻¹). The fluorescence lifetime of such chelated emissive species (A₂) is much larger and its contribution is also increased from 11% to 95% while the contribution of short lived component (A₁) corresponding to free probe molecules decreased from 89% to 5%. Hence, the data unequivocally corroborates ‘turn on’ sensing mechanism of FT towards Hg²⁺ ions via stable rigid complexation. The sensing mechanism is elaborated in Scheme 2.
Scheme 2. The proposed mechanism of Hg$^{2+}$ sensing by the probe FT.

Figure 13. Time-resolved fluorescence decay profiles of FT (20 µM) in presence and absence of Hg$^{2+}$ (5 equivalents) in ethanol-H$_2$O (9:1; 10 mM HEPES buffer, pH = 7.2); ($\lambda_{ex} = 340$ nm, $\lambda_{em} = 512$ nm); IRF (black); FT (pink); Hg$^{2+}$ (green).
Table 1. Photophysical parameters of sensor and sensor-Hg adduct.

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<td>0.057</td>
<td>0.296</td>
</tr>
</tbody>
</table>

2.12. Computational studies

To throw light on the behavior of ligand FT towards complexation with Hg<sup>2+</sup>, we performed ground state geometry optimization for the bare probe FT and its complexation adduct with Hg<sup>2+</sup> using B3LYP/6-31G(d,p) basis set in Gaussian 09 program.<sup>46,47</sup> The main optimized geometrical parameters for ligand and its complex are enlisted in Table 2 and 3 and the optimized ground state geometries are depicted in Figure 14.

![Figure 14. Ground state optimized geometry of FT and its complex FTM with Hg<sup>2+</sup> ions.](image-url)
Table 2. Selected optimized geometrical parameters of FT in the ground state calculated by DFT at B3LYP levels.

<table>
<thead>
<tr>
<th>Bond length (Å)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>N37-N38</td>
<td>1.398</td>
<td>N37-C35</td>
<td>1.404</td>
</tr>
<tr>
<td>N37-C5</td>
<td>1.520</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>N38-C39</td>
<td>1.304</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Bond angle (˚)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>C5-N37-N38</td>
<td>115.97</td>
<td>N37-N38-C39</td>
<td>120.061</td>
</tr>
<tr>
<td>C4-C5-N37</td>
<td>109.706</td>
<td>N37-C35-O36</td>
<td>125.977</td>
</tr>
<tr>
<td>C35-N37-N38</td>
<td>124.857</td>
<td>C27-C35-O36</td>
<td>128.580</td>
</tr>
</tbody>
</table>

Table 3. Selected optimized geometrical parameters for FTM in the ground state calculated by DFT at B3LYP levels.

<table>
<thead>
<tr>
<th>Bond length (Å)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>C34-N36</td>
<td>1.337</td>
<td>C34-O35</td>
<td>1.305</td>
</tr>
<tr>
<td>N36-N37</td>
<td>1.396</td>
<td>O35-Hg51</td>
<td>2.549</td>
</tr>
<tr>
<td>N37-C38</td>
<td>1.311</td>
<td>Hg51-O52</td>
<td>2.094</td>
</tr>
<tr>
<td>N37-Hg51</td>
<td>2.204</td>
<td>Hg51-O55</td>
<td>2.495</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Bond angle (˚)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>C5-C24-C26</td>
<td>122.189</td>
<td>N36-N37-C38</td>
<td>116.580</td>
</tr>
<tr>
<td>N37-Hg51-O52</td>
<td>151.548</td>
<td>N36-N37-Hg51</td>
<td>116.458</td>
</tr>
<tr>
<td>C26-C34-N36</td>
<td>116.428</td>
<td>N34-O35-Hg51</td>
<td>102.838</td>
</tr>
<tr>
<td>C34-N36-N37</td>
<td>114.627</td>
<td>N37-Hg51-O55</td>
<td>102.370</td>
</tr>
</tbody>
</table>

The structure of probe reveals that there is a large angle of twisting between the plane of 3-methylthiophenyl moiety and π-conjugated plane of fluorescein chromophore linked through hydrazone bridge. In the complex, Hg$^{2+}$ ion is four coordinated with fluorescein hydrazone and solvent water molecules in a distorted tetrahedral geometry with all the bond lengths fall in the region of 2.094-2.549 Å and bond angles in the range of 102.370°-151.548°.
In the case of FT, HOMO and LUMO orbitals mainly originate from π and π* orbital contribution of 3-methylthiophenyl linked hydrazone part with negligible electronic distribution on chromophore part, thus validating weak fluorescent property of ligand FT (Figure 15). The energy difference (band gap) between HOMO and LUMO in FT is calculated to be 4.05 eV. In the case of FTM, HOMO electron density is distributed over 3-methylthiophenyl conjugated hydrazone part whereas LUMO electron density is localized entirely on appended fluorescein chromophore supporting high fluorescent properties of the complex. The energy difference between HOMO and LUMO in FTM is calculated to be 2.66 eV which is much lower than that of the bare probe, revealing shifting of the emission to longer wavelength region. So, theoretical studies well corroborated substantial changes in fluorescence properties from ligand to complexed product.

Figure 15. The energy levels of frontier molecular orbitals of FT and FTM.

2.13. Logic Gate Device fabrication

The spectroscopic properties of the probe FT allows its fabrication as logic gate device. Owing to its reversible characteristics and binding properties, FT can exhibit an INHIBIT logic gate function and the output signal in the form of fluorescence emission at 512 nm is monitored by using Hg$^{2+}$ and Na$_2$EDTA as two inputs. The ‘turn-on’ fluorescence signal is
considered ‘1’ and ‘turn-off’ fluorescence signal is considered ‘0’ in the output of truth table as shown in Scheme 3 and Table 4.

![Scheme 3. Representation of an INHIBIT logic gate.](image)

**Table 4.** Corresponding truth table of the logic gate.

<table>
<thead>
<tr>
<th>In 1 (Hg^{2+})</th>
<th>In 2 (EDTA)</th>
<th>OUT (λem = 512 nm)</th>
<th>Intensity (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>FT = 173.3</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>1</td>
<td>FT+Hg = 541.5</td>
</tr>
<tr>
<td>0</td>
<td>1</td>
<td>0</td>
<td>FT+EDTA = 200.8</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>0</td>
<td>FT+Hg+EDTA = 150.8</td>
</tr>
</tbody>
</table>

2.14. Real water sample analysis

To analyze the practical applicability of the sensor FT, real water sample analysis was performed for determination of as low as of nano-molar level of Hg^{2+} ion. Initially, a calibration curve was constructed by plotting the maximum emission intensity with Hg^{2+} concentration (0–80 nM) in only HEPES buffer (20 mM, pH = 7.2) distilled water medium (ESI, Figure S12). A linear equation was observed with a correlation coefficient of R^2 = 0.991. Then the field water samples (tap water) were tested without any prior treatment. A standard addition method was adopted to establish the recovery of Hg^{2+}, the water samples were spiked with a known concentration of Hg^{2+} (50 nM). To confirm this result the field samples were measured in absence and presence of Hg^{2+} by atomic absorption spectrophotometer (AAS). All the results are summarized in Table 5 with a negligible error (0.5–1.6%), indicates an outstanding agreement between the values found in AAS method and our method. Therefore the receptor FT can undoubtedly be used to determine Hg^{2+} in environmental samples with insignificant interference.
Table 5. Determination of Hg\(^{2+}\) in water samples (Sample specification: Sample A- Bankura, Sample B- Durgapur, Sample C- Purulia)

<table>
<thead>
<tr>
<th>Source of water sample</th>
<th>Amount of spiked Hg(^{2+}) (nM)</th>
<th>Hg(^{2+}) found in fluorescence spectrometer (nM)</th>
<th>% of Deviation</th>
<th>Hg(^{2+}) found in AAS (nM)</th>
<th>% of Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample A</td>
<td>0.00</td>
<td>0.00</td>
<td>-</td>
<td>0.00</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>50.379</td>
<td>1.3</td>
<td>49.412</td>
<td>0.8</td>
</tr>
<tr>
<td>Sample B</td>
<td>0.00</td>
<td>0.00</td>
<td>-</td>
<td>0.00</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>50.197</td>
<td>0.9</td>
<td>49.631</td>
<td>0.5</td>
</tr>
<tr>
<td>Sample C</td>
<td>0.00</td>
<td>0.00</td>
<td>-</td>
<td>0.00</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>50.561</td>
<td>1.6</td>
<td>49.113</td>
<td>1.4</td>
</tr>
</tbody>
</table>

3. Conclusions

In a nutshell, a highly facile, rapid, sensitive and reversible thiophene appended fluorescein-hydrazone chemosensor was reported for selective screening of Hg\(^{2+}\) ions in aqueous medium. The proposed sensor FT showed the detection limit as low as 137 nM. Various steady state and time-resolved spectroscopic analysis as well as DFT based theoretical studies confirmed the complexation of the probe FT with Hg\(^{2+}\). Moreover, the proposed FT-Hg\(^{2+}\) complex was found to be reversible in presence of EDTA and thus making FT-Hg\(^{2+}\) complex an efficient sensor for EDTA. The theoretical studies well corroborated substantial changes in fluorescence properties from ligand to complexed product. The potential applications of the proposed sensor were also demonstrated in both real time quantitative detection of Hg\(^{2+}\) ions in environmental real water samples and logic gate based device fabrication for future incorporation in small organic molecule based efficient molecular devices.

X-ray crystallography

Deposition Number 2095599 contains the supplementary crystallographic data for this paper. These data are provided free of charge by the joint Cambridge Crystallographic Data Centre and Fachinformationszentrum Karlsruhe Access Structures service www.ccdc.cam.ac.uk/structures.

Supporting Information Summary

Experimental procedure, characterizations (SCXRD, HR-MS, FT-IR), analytical and theoretical data are provided in the Supporting Information.

Acknowledgement

The authors thankfully acknowledge DST-FIST (SR/FST/CSI- 267/2015 (C)) and NIT Durgapur for creating and providing infrastructural facilities for research. The authors are thankful to IICB Kolkata for the HR-mass spectra analysis. SSP also acknowledge the financial support from DST SERB (EMR/2016/001230 dt.15.03.2017).
Keywords: Chromo-fluorogenic sensor, Hg(II) recognition, PET, Reversible binding, Time-resolved fluorescence spectroscopy.

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Staroverov, R. Kobayashi, J. Normand, K. Raghavachari, A. Rendell, J. C. Burant, S.
Table of Contents

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A new thiophene appended fluorescein-hydrazone derivative (FT) has been demonstrated as real-time sensory system for Hg$^{2+}$ ions in aqueous medium. The detection limit of the probe FT towards Hg$^{2+}$ was 137 nM. The FT-Hg$^{2+}$ complex was found to be reversible in presence of EDTA. Significance of the probe lies in its successful application for the detection and quantification of Hg$^{2+}$ in real water samples and logic gate fabrication for future incorporation in small organic molecule based efficient molecular devices.