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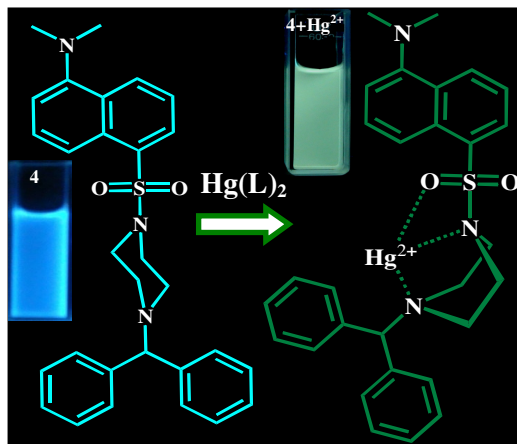
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Graphical Abstract



**A simple blue fluorescent probe to detect Hg²⁺ in semiaqueous environment
by intramolecular charge transfer mechanism**

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An efficient intramolecular charge transfer fluorescent probe bridging benzhydryl moiety and dansyl fluorophore through piperazine unit has been synthesized and characterized. The photophysical behavior of synthesized probe has been analyzed in the presence of different cations in aqueous acetonitrile solution. The probe has shown sensitivity to detect Hg²⁺ ion selectively over other tested cations at 20 nM level. The probable mode of binding of Hg²⁺ through the nitrogen and oxygen atoms of piperazine and dansyl has been established by spectral data analysis.

Keywords: Fluorescence, Hg²⁺, ICT

Over the last few decades, among the heavy and transition metal (HTM) ions Hg^{2+} is considered as most toxic heavy metal ion and common pollutant that possesses severe risks for human health and environment.^{1,2} Mercury is commonly spread in environment by anthropogenic and industrial releases and is converted to toxic methylmercury by the bacterial and chemical actions. The bioaccumulation of such toxic stuff in living tissues of human and animal bodies via a food chain causes mercury poisoning and lethal diseases.^{1,2} Environmental Protection Agency (EPA) has set maximum allowable level of inorganic mercury in drinking water 2 ppb.³ Considerable efforts have been devoted to develop chemosensors for Hg^{2+} and among several methods fluorescence based detection methods have been found more reliable, inexpensive, sensitive, easy to perform, and are suitable for real time monitoring of anions and metal ions.⁴ However, poor aqueous medium compatibility⁵ and nonspecific fluorescence quenching induced by HTMs due to enhanced spin-orbit coupling^{6a} and electron transfer mechanism^{6b,c} are kind of limitations for such sensing systems to become a good analytical tool. Therefore, a sensitive fluoroionophore compatible with aqueous or partial aqueous medium and which can show characteristic optical behavior upon interaction with metal ions particularly, Hg^{2+} is highly demanding.⁷

Currently, fluorescent probes has attracted considerable interest because of their intrinsic sensitivity and selectivity.⁸ In this direction several efforts have been made to design efficient fluorescent molecular sensors⁹ to detect Hg^{2+} either by fluorescence enhancement or quenching and color changes.^{10,11} The major challenges in the development of a sensitive Hg^{2+} sensor are due to silent optical spectroscopic signature of Hg^{2+} because of closed-shell d^{10} configuration, a background of competing analytes¹² and compatibility in an aqueous or partial aqueous media. Many reports dealing with fluorescent probes for instance, azacrown ethers,^{13a} dithioaza compounds,^{13b} calixarene-based ionophores,^{13c-d} DNA,^{14a} MerR family proteins,^{15a} fluorescent dye doped crystalline complexes,^{15b} and so on^{15c} have appeared in literature to detect Hg^{2+} ions however, only a few of them are found competitive¹⁴ in terms of sensitivity, selectivity, and measurements in aqueous media. Therefore, for Hg^{2+} design and synthesis of new systems based on small organic molecular systems and/or scaffolds that are cost-effective, rapid, facile and applicable to the environmental and biological milieus is an important goal to provide instant optical feedback without involving sophisticated instrumentation.

Keeping these perspective in mind our ongoing research is currently paying attention toward development of some efficient fluorescent organic scaffolds/motifs to detect cations, anions, and biomolecules sensitively in different media.¹⁶ Through this contribution we present design and synthesis of a new class of benzhydryl piperazine dansyl conjugate, **4** and its potential application to detect Hg^{2+} in a partial aqueous medium. While synthesizing such typical motif we considered that soft metal ions like, Hg^{2+} has affinity for ligands containing nitrogen as a donor side. Simple heterocycles like, piperazine, a strong base (pK_a , 9.8) than pyridine (pK_a , 5.2) and aniline (pK_a , 4.6) will favor a stable 1:1 complexation with mercuric salts in aqueous medium.¹⁷ Also, introduction of benzhydryl moiety in conjugation to piperazine and dansyl would generate a stable configuration by acquiring an appropriate amount of energy and become an efficient electron rich charge transfer probe to work on push-pull mechanism.^{2,8}

Scheme 1 illustrates the synthesis and structure of probe, **4**. Benzophenone upon reduction with sodium borohydride in methanol gave biphenylmethanol, **1** which was converted to biphenylmethylchloride, **2** in good yield by reacting with thionyl chloride. Piperazine dihydrochloride and compound **2** in the presence of potassium carbonate were reacted under refluxing condition in THF to afford 1-benzhydrylpiperazine, **3** in 80% yield. Subsequently, **3** treated with dansyl chloride in THF under anhydrous condition to yield benzhydryl piperazine dansyl conjugate, probe **4** as a light yellow color powder in ~75% yield. All products were characterized by ¹HNMR, FTIR, and ESI-MS spectral data analysis (See supplementary information, Figure S1-S6).

Dansyl is sensitive to variation in external environment and a slight variation in the medium would result a dramatic change in its typical fluorescence behavior attributed to intramolecular charge transfer (ICT) reaction from *N,N'*-dimethylamine ($-N(\text{CH}_3)_2$) to sulfonamide function.¹⁸⁻²⁰ Also, dansyl appended probes show solvatochromic nature (sensitivity to polarity of the medium due to a change in fluorophore's dipole moment on optical excitation in solvents of different dielectric constants and refractive indexes) owing to the presence of locally excited (LE) and twisted intramolecular charge transfer (TICT) excited state.^{21a,22a} So, upon excitation, first a less polar semi-planar state, similar in structure to ground state is formed to exhibit structured emission (LE). This LE state undergoes ICT from *N,N'*-dimethylamino (the donor) to the sulfonamide fragment (the acceptor) with a twist along the bond connecting the donor and acceptor units. That creates a relatively more polar TICT state in which donor and acceptor units

become perpendicular to each other. The emission corresponding to TICT state is structureless and appears at higher wavelength in comparison to LE state. The TICT state is sensitive to solvent polarities which help to mediate CT. Therefore, high energy fluorescence is associated with LE while low energy fluorescence is through TICT state. The polarity of the solvent up to a certain extent also governs the height of the energy barrier for the conversion of LE to ICT as well as stabilize the ICT state. Therefore, when the ICT behavior of **4** has been studied in solvents of different dielectric constants such as, hexane, chloroform, benzene, acetonitrile, methanol and dimethylformamide the emission maxima appeared at different wavelengths. The red shift observed in emission band along with increase in Stoke's shift were found in the order as; Hexane < benzene < chloroform < acetonitrile < dimethylformamide < methanol (Figure S7 and Table S1).

Further, the optical behavior of present fluorescence motifs **4** has been examined at different pH by absorption and emission spectroscopy (Figure 1). In the alkaline medium (pH 7 to 14) absorption spectra showed a slight enhancement in the absorption band centered at 342 nm ($\epsilon = 5.8 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$) while, in acidic medium (pH 6 – 1) band centered at 342 nm decreased gradually and a new blue shifted band of relatively high extinction coefficient ($\epsilon = 1.0 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$) appeared at 286 nm (Figure 1a). Similarly, emission spectra of **4**, in alkaline medium (pH 7 to 14) showed insignificant change in fluorescence intensity at 540 nm. However, in acidic pH 6 – 1 fluorescence intensity at 540 nm decreased gradually (80%) and a new emission band appeared at -400 nm (Figure 1b). The variation in the optical behavior of **4** at different pHs may be attributed to TICT processes¹⁸ wherein under the acidic conditions decrease in emission intensity with a large blue shift is probably due to the protonation of $-N(CH_3)_2$ function and of piperazine unit (high pK_a , 9.8), ultimately restricting typical ICT reaction.¹⁸ The pH studies also suggested about the applicability of **4** to work around physiological pH 7. Moreover, when the pH studies have been performed in HEPES buffer almost similar optical pattern was observed (Figure S8). Thus, the above experimental observations clearly suggest about the ICT process in the studied probe **4**.

The photophysical behavior of **4** in the absence and presence of metal ions has been examined in aqueous-acetonitrile (H_2O -MeCN; 50%) through absorption and emission spectrometry. It is expected that MeCN or MeCN- H_2O gradient system would facilitate the TICT process. The absorption spectrum of **4** (10 μM) show characteristic intramolecular charge transfer band at 342

nm ($\epsilon = 58000 \text{ M}^{-1} \text{ cm}^{-1}$) (Figure 2, S9). Upon excitation at 342 nm **4** displayed a weak emission band centered at 540 nm with Stoke's shift of 10721 cm^{-1} . The ability of **4** to interact with different cations has been examined by the addition of different metal ion (5 equiv) namely, Na^+ , K^+ , Ca^{2+} , Zn^{2+} , Pb^{2+} , Ag^+ , Cd^{2+} , Co^{2+} , Ni^{2+} , Cu^{2+} and Hg^{2+} to a solution of **4**. The absorption spectra in the presence of metal ions do not show any significant modulation except, a marginal change with Hg^{2+} ion in which, absorption band centered, at 342 nm enhanced marginally ($\epsilon = 0.03\%$). Further, the fluorogenic affinity of **4** ($1 \mu\text{M}$) toward tested metal ions (5 equiv) has been analyzed through emission spectra, at 342 nm excitation. The emission spectra revealed local and TICT excited states at ~ 400 and 540 nm respectively. The emission band centered at 540 nm showed fluorescence quenching selectively with Hg^{2+} in which the relative fluorescence intensity of **4** decreased, $\sim 30\%$ (Figure 3a). The competitive metal ions interference experiment when performed by the addition of excess of tested cations (20 equiv) to a solution of complex, **4**- Hg^{2+} revealed insignificant change in the absorption and emission spectra of **4**- Hg^{2+} thus, suggesting high sensitivity of **4** toward Hg^{2+} ion selectively (Figure 2b, 3b, S9). To ascertain that the process of complexation between **4** and Hg^{2+} is reversible a strong chelating reagent, EDTA was added, in excess to a solution of **4**+ Hg^{2+} . The fluorescence intensity revived and was found almost close to the intensity of **4** (Figure S10a) thus, suggested about the reversible mode of complexation between **4** and Hg^{2+} ions. In contrast, when Hg^{2+} ions were added to a solution of **4** containing EDTA in excess, emission spectra show insignificant change probably due to the formation of a strong EDTA- Hg^{2+} complex in the medium (Figure S10b).

The binding affinity of **4** with Hg^{2+} has been determined by absorption titration studies (Figure S11a). Upon a gradual addition of Hg^{2+} (0-15 equiv.) absorption band centered, at 342 nm enhanced marginally ($\epsilon = 0.03 \%$). However, excess of Hg^{2+} ions (20-50 equiv.) was added to the same solution ICT band decreased almost completely ($\epsilon = 0.82 \%$) and a new band appeared at 288 nm ($\epsilon = 1.8 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$). That may be attributed to either protonation at $-\text{N}(\text{CH}_3)_2$ function^{22b} and/or interaction of Hg^{2+} with $-\text{N}(\text{CH}_3)_2$ function of dansyl^{16b} as well as nitrogen atoms of piperazine unit.¹⁷ To confirm the possibilities of interactions absorption titration experiment was performed at pH 7 in HEPES buffer (10 mM; H_2O -MeCN; 50%). Upon a gradual addition of Hg^{2+} (0-15 equiv) ICT band centered at 342 nm increased marginally ($\epsilon = 6 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$) with a blue shift of 4 nm . However, upon increasing concentration of Hg^{2+} ions (20-50 equiv.) absorption spectra showed insignificant change (Figure S11b). This clearly

suggested that Hg^{2+} is not interacting with $-N(\text{CH}_3)_2$ function of dansyl at pH 7 while in aqueous-ACN (50%) it could have happened probably because of acidic pH of medium generated by the addition of excess of mercury as its nitrate salt.

The binding affinity of **4** with Hg^{2+} ions has been determined by emission titration studies. Upon a gradual addition of Hg^{2+} (0 - 15 equiv) to a solution of **4** the emission spectra displayed, ~ 81% fluorescence quenching, in which relative fluorescence intensity of emission band centered at 540 nm decreased gradually with a bathochromic shift of 20 nm and the naked-eye sensitive color of solution changed from a bright fluorescent blue to a bluish-green (Figure 4). The estimated quantum yield^{21a} of **4** ($\Phi_4 = 0.053$, in MeCN/H₂O) upon interaction with Hg^{2+} ions decreased to 0.012 ($\Delta\Phi_{4 + \text{Hg}^{2+}} = 0.041$). The resultant Job's plot revealed a 1:1 stoichiometry for **4** - Hg^{2+} interaction (Figure 5a). The binding constant for a 1:1 stoichiometry calculated from Benesi-Hildebrand (*B-H*) method^{21b} utilizing emission titration experiment data, gave $K_{ass} = 3.3 \times 10^5 \text{ M}^{-1}$ (Figure 5b). The extent of fluorescence quenching was estimated quantitatively by obtaining Stern-Volmer (*S-V*) plot between concentration of Hg^{2+} ions and change in relative fluorescence intensities (ΔI). An almost straight line *S-V* plot with quenching constant, $K_{s-v} = 2 \times 10^5 \text{ M}^{-1}$ (Figure 5c) suggested fluorescence quenching in the present sensing event. The sensitivity (LOD) of **4** to detect Hg^{2+} in H₂O-MeCN solution has been estimated by our previously reported method^{16a,b} and was found to be 20 nM (4 ppb) which is low, (Figure 6) but comparable to other reported methods.^{16a,k}

Moreover, to confirm the interaction of Hg^{2+} emission titration experiment was repeated at pH 7 in HEPES buffer (Figure S12). The emission spectra revealed local and TICT excited states and upon addition of Hg^{2+} fluorescence quenching occurred in which intensity of CT band centered at 540 nm decreased with a bathochromic shift of 12 nm without generation of any additional emission band corresponding to the protonation of $-N(\text{CH}_3)_2$ function. Furthermore, the pH-emission spectra of **4** showed that from pH 7 to 5 the CT emission decrease with a marginal rise in intensity of band centered at 400 nm in HEPES buffer (Figure S13a). However at pH 4 the low energy band decreased abruptly and high energy band become prominent. This is attributed to protonation of $-N(\text{CH}_3)_2$ function of dansyl as well as piperazine unit. Upon addition of Hg^{2+} at pH 4.0, insignificant change in the emission spectra of **4** was observed (Figure S13b) which clearly suggested about the interaction of Hg^{2+} with nitrogen atoms of piperazine unit rather than interaction with dansyl fluorophore.

Additionally, the mechanism of fluorescence quenching either by charge/electron transfer or energy transfer can be determined thermodynamically by performing spectrofluorometric investigations on a frozen solution at liquid nitrogen temperature.^{19b,23} In a frozen matrix, immobilization of the solvent molecule prevents solvent reorganization surrounding the generated charge separated molecules and raises the energy of the ion-pair species and prevent electron transfer. Consequently, if the electron transfer quenching mechanism prevails at room temperature, freezing of the solution at 100K would partially or fully restore the fluorescence emission. To observe that when the emission spectra of the probable complex, **4**+Hg²⁺ was acquired in EtOH/MeOH solvent mixture (9:1, v/v) and fluorescence quenching (~73%) was observed with a bathochromic shift of ~ 16 nm (Figure 7). In contrast on a frozen solution (rigid glass matrix, at ~100K), the emission of complex restored partially (49%). Thus, the experimental observations clearly suggesting about quenching mechanism through a charge/electron transfer process.^{19b,23}

Further insight about actual mechanism of interaction between **4** and Hg²⁺ were followed by ¹H NMR titration studies. The ¹H NMR spectrum of **4** in DMSO-*d*₆ (Figure 8) showed three doublets at δ 8.53-8.08 ppm assigned to of H2, H8 and H4 protons while multiplet at δ 7.68-7.54 ppm to H3 and H7 proton resonances of naphthyl ring while H6 and benzhydryl ring protons resonates at δ 7.39-7.11 ppm. The resonances appeared at δ 4.24, 3.02 and 2.29 ppm can be assigned to methyl protons, -CH (1'') and -CH₂ (2' and 1') of benzhydryl and piperazine rings respectively, and signal at δ 2.82 ppm is attributable to protons of -N(CH₃)₂ function. Upon a gradual addition of Hg²⁺ ions (0 – 2 equiv) to a solution of **4** -CH (1'') resonances shifted downfield (Δδ = 1.1 ppm) and emerged at δ 5.34. The -CH₂ (1') proton of piperazine ring showed significant downfield shift, Δδ = 0.71 ppm to appear at δ 3.01 ppm and while -CH₂ (2') shifted downfield, Δδ = ~ 0.26 ppm and merged with signal of DMSO-*d*₆. The resonances attributed to -N(CH₃)₂ protons showed almost negligible change whereas the benzhydryl and naphthalene ring protons shifted downfield narrowly in the range of δ 0.03-0.12 ppm. Thus, the change in the spectrum of the **4** upon addition of Hg²⁺ ions suggested about the possibility of interaction of Hg²⁺ with piperazine ring nitrogen atoms well stabilized with oxygen atom of sulfonamide function. Moreover, it is reasonable to assume that upon interaction with Hg²⁺ ions the orientation of piperazine unit undergo some structural changes, in which the chair form is flipped to a boat form. A 1:1 binding equilibrium between **4** and Hg²⁺ ions and insignificant change in the

resonances of $-N(CH_3)_2$ function of dansyl fluorophore ruled out the possibility of interaction through $-N(CH_3)_2$ unit as demonstrated previously.^{16a,24} Therefore, the observed fluorescence quenching upon interaction with Hg^{2+} is probably due to the transfer of electron²¹ from the excited state of the fluorophore to the available d – orbital of Hg^{2+} precisely, due to strong spin-orbit coupling²⁵ enforced by Hg^{2+} on the probe.

Further, the probable complexation between **4** and Hg^{2+} ion was analyzed through FT-IR spectra measured in range 4000–400 cm^{-1} (Figure S14). The FTIR spectrum **4** showed characteristic bands at 2801, 1660 1492, 1447 1340 846 and 1281 1137 cm^{-1} probably due to C-H, C=C, C-N and S=O stretching vibrations. Similarly, bands appeared at 1000, 744, 705 and 805 cm^{-1} may be assigned to C-S, C-H (aromatic) and S-O (sulfonamide) stretching vibrations, respectively.^{16e,1} Upon interaction with Hg^{2+} ions, C-N and S=O stretching vibrations shifted to appear at lower frequencies at 1434, 1329, 794 and 1143, 1110, 1087 cm^{-1} . The C-S stretching vibrations also showed a shift in lower frequency region, at 916 and 707 cm^{-1} . The stretching vibrations appeared at 626, 569 and at 482 cm^{-1} are possibly due to the M–O and M–N (Hg -O/N) interactions.^{16e,1} Therefore, supporting about the involvement of nitrogen and oxygen atoms of piperazine and sulfonamide units, as a potential donor site to form a complex, **4**- Hg^{2+} as predicted in Scheme 1. The ESI-MS spectral data (Figure S6 and 15) analysis of **4** before and after interaction with Hg^{2+} [$(HgClO_4)_2$] revealed m/z at 486 (**4** +H)⁺ and at 787.8 [**4** + Hg + ClO_4 + H]⁺ supported the mode of binding between **4** and Hg^{2+} involving the N and O atoms of piperazine and sulfonamide oxygen of dansyl.²⁶

In summary, through the present contribution we have demonstrated the application of a new charge transfer blue fluorescent probe to detect Hg^{2+} selectively in a partial aqueous medium with sensitivity of detection up to 20 nM. The optical behavior of probe and NMR spectral data analysis suggested about the interaction of probe with Hg^{2+} ion through the nitrogen atoms of piperazine unit well stabilized by oxygen atom of sulfonamide function.

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Supplementary data: Synthesis, ¹HNMR, ¹³CNMR, FT-IR, ESI-MS, UV-Vis and fluorescence spectra.

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Figure Captions

Scheme 1: Synthesis of fluorescent probe **4** and plausible mode of complexation with Hg^{2+} ion. (i) NaBH_4 / Ethanol/ rt (ii) SOCl_2 / DCM / rt (iii) Piperazine / K_2CO_3 / DMF/ reflux / 8h (iv) Dansyl chloride / Acetone / rt

Figure 1. Change in (a) absorption and (b) emission spectra of **4** at different pH in aqueous ACN (50%).

Figure 2. (a) Change in absorption spectra of **4** (10 μM) upon interaction with different metal ions. (b) Interference study by the addition of different cations to a solution of **4**- Hg^{2+} in HEPES buffer (10 mM; aqueous ACN (50%) pH 7).

Figure 3. Change in emission spectra of **4** (10 μM) (a) upon interaction with different metal ions and (b) Interference study by the addition of different cations to a solution of **4**- Hg^{2+} in aqueous ACN (50%). Inset: Bar diagram of interaction study.

Figure 4. Emission titration spectra of **4** upon addition of Hg^{2+} (0- 15 equiv) in aqueous-acetonitrile (50%). Inset: Change in color of **4** under UV light.

Figure 5. (a) Jobs plot (b) B-H plot (c) Stern-Volmer plot of **4** and Hg^{2+} ions in aqueous ACN (50%).

Figure 6. (a) Calibration curve for **4** and (b) Calibration sensitivity of Hg^{2+} ions in aqueous ACN (50%).

Figure 7. Emission spectra of **4** and **4**+ Hg^{2+} at normal temperature and revival of fluorescence at liquid nitrogen temperature.

Figure 8. ^1H NMR titration spectra of probe **4** upon addition of Hg^{2+} ions (0-2 equiv.) in $\text{DMSO-}d_6$.

**A simple blue fluorescent probe to detect Hg²⁺ in semiaqueous environment
by intramolecular charge transfer mechanism**

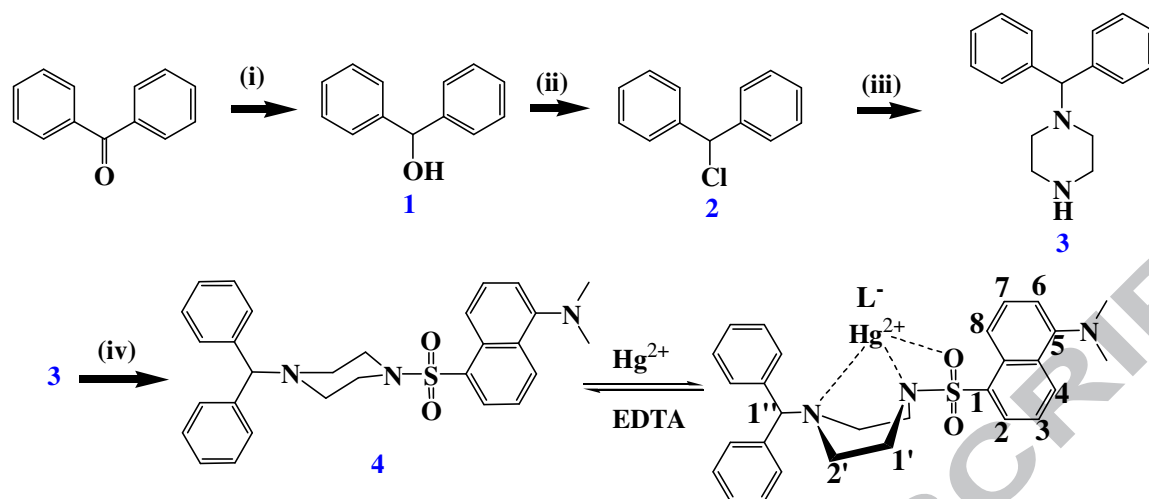
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ACCEPTED MANUSCRIPT



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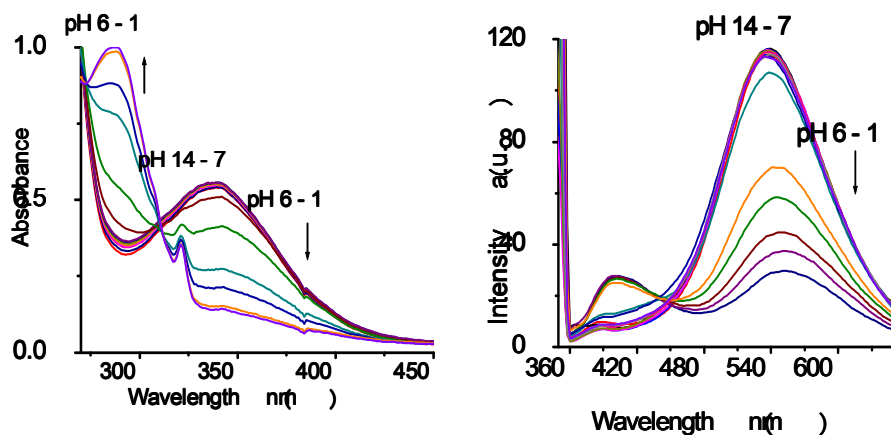


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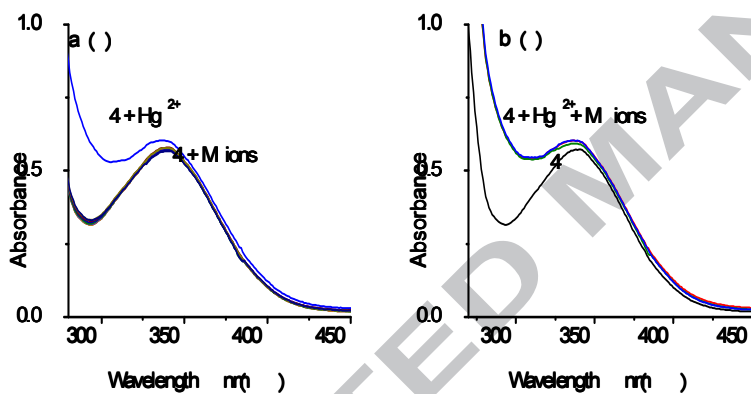


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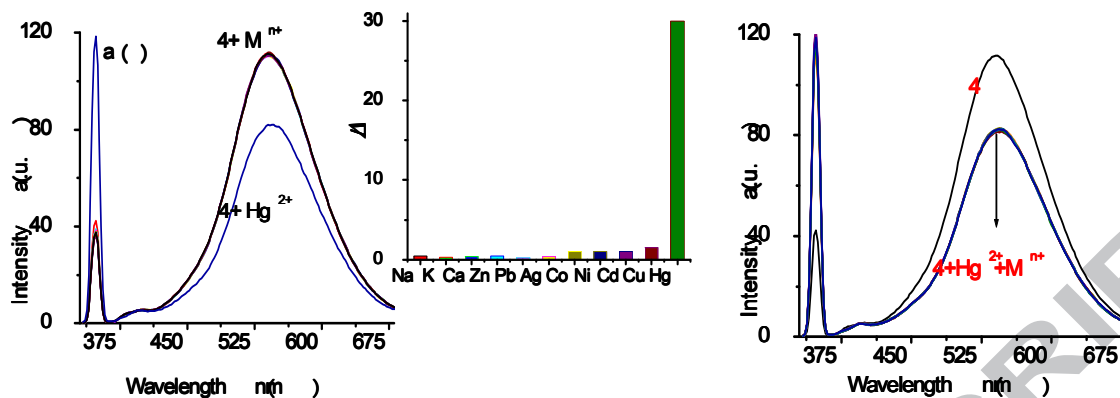


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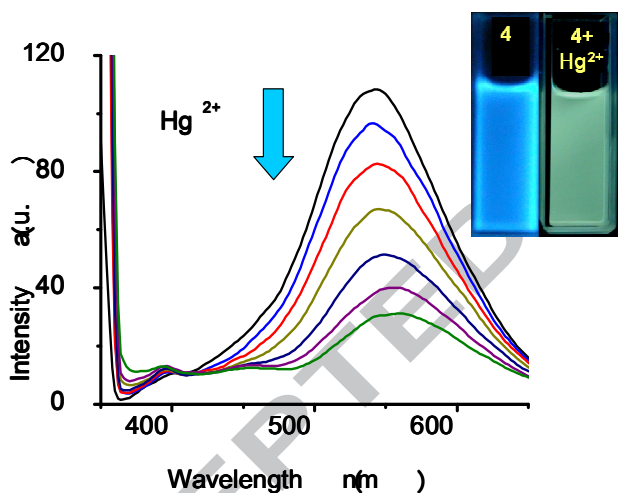


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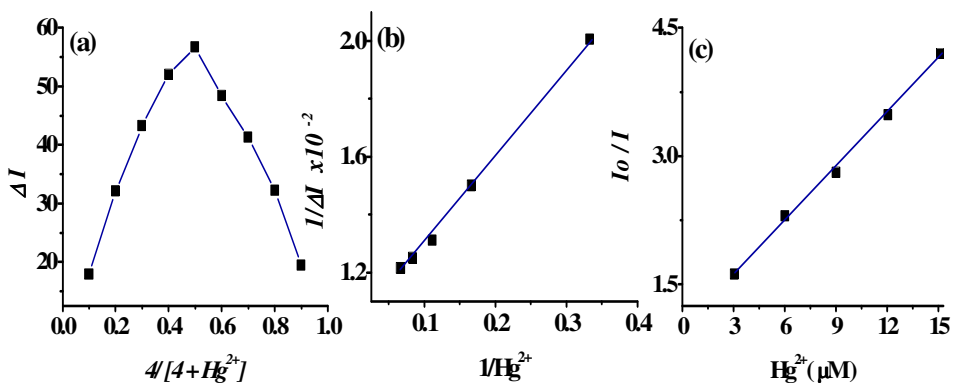


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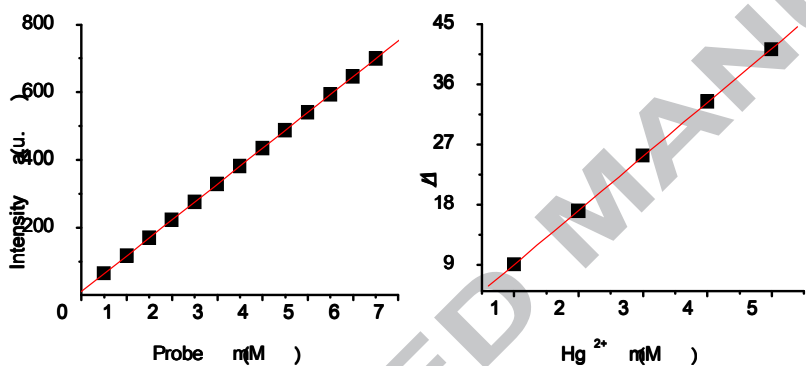


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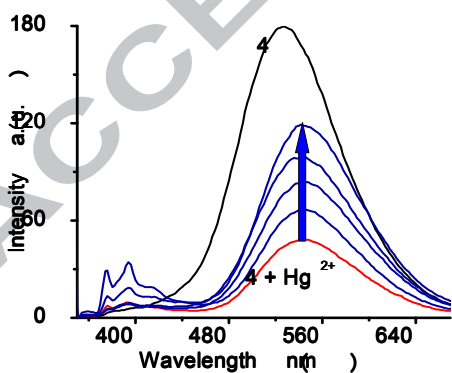


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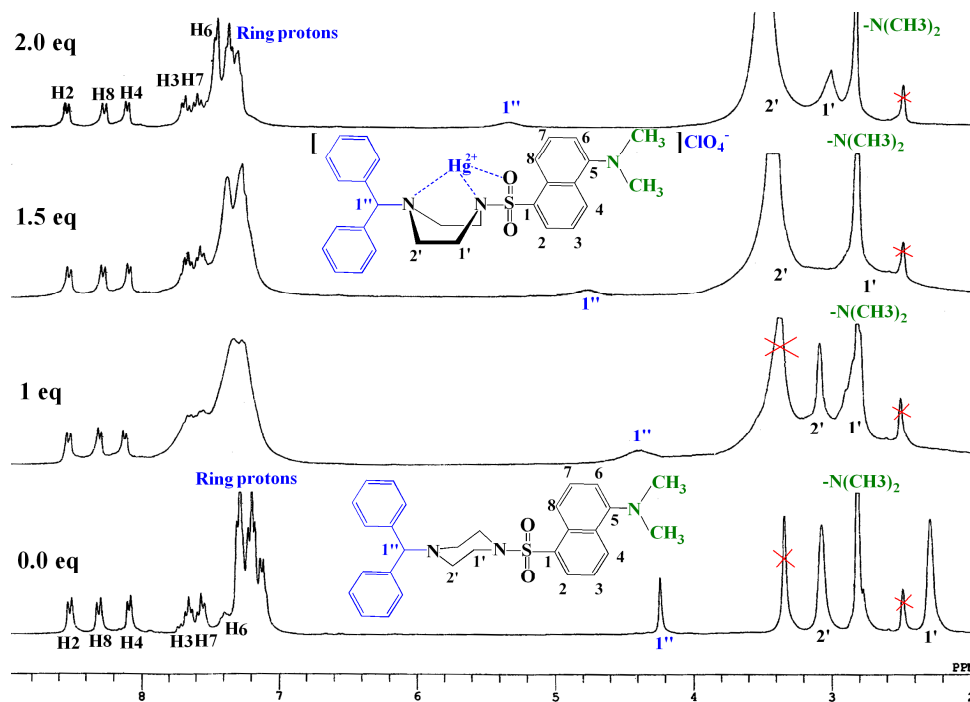


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