# Azine-Based Receptor for Recognition of Hg²⁺ Ion: Crystallographic Evidence and Imaging Application in Live Cells

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ABSTRACT
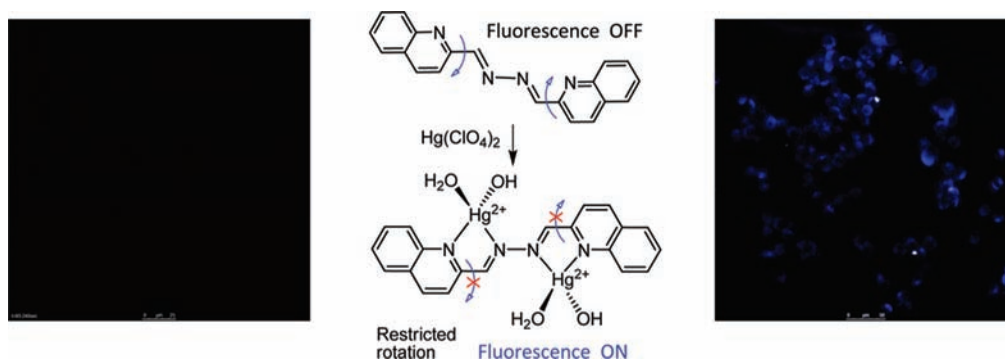
A newly synthesized azine-based receptor (L) is found to show remarkable specificity toward the Hg²⁺ ion in aqueous media over other metal ions. Coordination of L to Hg²⁺ induces a detectable change in color and a *turn-on* fluorescence response. Restricted C=N isomerization of the azine moieties in the excited state as well as the Photoinduced Electron Transfer (PET) involving the lone pair of electrons of N¹/N² on coordination of L to the Hg²⁺ ion account for the *turn-on* fluorescence response. This reagent could be used for imaging the accumulation of Hg²⁺ ions in Epithelial cell line KB 31 cells.

Mercury is one of the most toxic heavy metals and has significant industrial and agricultural uses. These uses have led to severe localized mercury pollution in the atmosphere, lithosphere, and surface water. Inorganic mercury is converted to the organic species of mercury, which are accumulated in the tissues of higher organisms. This causes systemic diseases and triggers various neurological disorders, such as Minamata disease, prenatal brain damage, etc.[1] Despite efforts to curb the mercury emission in the environment, the global mercury contamination from natural processes such as oceanic and volcanic emission,

coal-burning, gold mining, and solid inceneration has posed a big threat to the human race in the last few decades.[2] The concern over its deleterious effects on human health has actually led the chemistry and, more broadly, the sensing community to develop new mercury detection methods that are cost-effective, rapid, facile, and applicable to the environmental and biological milieus.[3] In this regard, receptor molecules that can provide optical feedback on binding to the Hg²⁺ ion in aqueous or mixed aqueous environments in the form of visually detectable change in color[4] and fluorescence[5] are expected to find applications both as colorimetric staining agents for easy and facile detection and as fluorescent imaging reagents for the biological cells that are affected with Hg²⁺ ion adsorption. Such

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examples of receptor molecules are not very common in the literature, and importantly, receptor molecules that show enhancement on binding to the Hg^{2+} ion are even more scarce.[3] Being one of the heavier metal ions, the Hg^{2+} ion is commonly known to quench the fluorescence effectively due to an efficient spin−orbit coupling.[6] However, any optical feedback that translates the metal ion−receptor binding phenomena into a *fluorescence turn-on* response is preferred over the *fluorescence turn-off* response for obvious ease in the detection process(es). Thus, any sensor molecule that works on the basis of *fluorescence turn-on* response, as well as can be used for colorimetric detection of Hg^{2+} in aqueous or mixed aqueous−organic solvent medium, is important from the viewpoint of its application potential as a staining or imaging reagent for biological applications.[7]

In this article, we report a quinoline-based sensor (**L**) having an azine-type receptor functionality for specific recognition of the Hg^{2+} ion in mixed aqueous−organic solvent medium. An earlier report[3e,10b] on the possibility of the analogous bisazine-type ligand to act as a bischelating ligand toward transition metal ions has led us to synthesize the receptor **L** for specific recognition of Hg^{2+}. More importantly, the possibility of using this reagent for detection of Hg^{2+} accumulated in Epithelial cell line (KB 31 cells, preexposed to Hg^{2+} solution) using confocal laser microscopy is also reported.

The reagent **L** was synthesized following a one-step reaction between quinoline-2-carbaldehyde and hydrazine hydrate in 85% yield (Supporting Information). Various analytical and spectroscopic data agree well with the structure proposed for this reagent and the desired purity for further use (Supporting Information).

The absorption spectra of **L** in THF/H$_2$O (6:4, v/v) showed two main absorption bands with λ_{max} of 265 and 330 nm. These transitions could be accounted for $^1L_a \leftarrow {}^1A$ and $^1L_b \leftarrow {}^1A$, respectively. Addition of a perchlorate salt of alkali, alkaline earth, and common transition metal ions (Li^+, Na^+, K^+, Cs^+, Ca^{2+}, Mg^{2+}, Sr^{2+}, Ba^{2+}, Cr^{3+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}) did not show any change in spectra of **L**. However, an appreciable change in the electronic spectra for **L** was observed in the presence of Hg^{2+}; a new absorption band with maximum at 370 nm was observed (Figure 1).
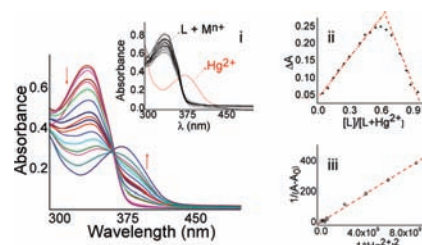
Figure 1. Changes in UV−vis spectra of **L** (8.0×10^{-5} M) in THF/H$_2$O (6:4, v/v) with varying [Hg^{2+}]. Insets: (i) UV−vis scanning spectra of **L** (8.0×10^{-5} M) in the presence of various metal ions. (ii) Job's plot for evaluation of the binding stoichiometry between $Hg(ClO_4)_2$ and **L** in THF/water (4:6, v/v) medium. (iii) Benesi−Hildebrand plot for evaluation of the binding constant and linearity of this plot with $R^2 = 0.99$, which also confirms the 2:1 binding stoichiometry.

Use of organic solvent was unavoidable due to limited solubility of **L** in water. Systematic spectrophotometric titration with increasing [Hg^{2+}] revealed a gradual decrease of absorption band at 330 nm with the concomitant increase in the new charge transfer transition band at 370 nm (Figure 1). The formation of this new charge transfer band with a clear isosbestic point at 359 nm suggests that the **L** and **L** bound to Hg^{2+} ions exist in equilibrium. The stoichiometry for the binding of **L** to Hg^{2+} was determined using Job's plot method and was found to be 2:1—suggesting the formation of a complex such as $[(Hg^{2+})_2L]^{4+}$. Formation of $[(Hg^{2+})_2L]^{4+}$ seems logical, as bisazine ligands analogous to **L** are expected to act as a bischelating ligands.[8] Further, such a binding stoichiometry was also confirmed from the ESI−MS spectral data, the signal for [L + 2Hg + Na]$^+$ at 732.57 and [L + 2Hg + 2ClO$_4$]$^+$ at 908 with the anticipated isotope distribution signifying the $[(Hg^{2+})_2L]^{4+}$ (Supporting Information).

The equilibrium constant for the formation of $[(Hg^{2+})_2L]^{4+}$ from the interaction of **L** and Hg^{2+} was calculated from the plot $1/[A − A_0]$ vs $1/[Hg^{2+}]^2$ using the Benesi−Hildebrand equation[9] (Supporting Information) and was found to be 2.37×10^7 M^{-2} L^2. Linearity of the plot for 1:2 binding also confirms this binding stoichiometry.

The absence of any other equilibrium process reveals that either the binding of two Hg^{2+} ions to **L** is a simultaneous

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process or the difference between two successive binding constants is too narrow to be resolved. Thus, the observed binding constant could be better assigned as the composite binding constant for the formation of $\{(Hg^{2+})_2L\}(ClO_4)_4$.

The emission spectrum of L in THF/H$_2$O (6:4, v/v) was recorded following excitation at 330 nm. Emission intensity for L was found to be very low; weak emission bands with maxima at 376 and 406 nm were observed. Flexibility and rapid C=N isomerization of imine or azine functionality, coupled to a quinoline fragment, account for non-radiative deactivation of the quinoline-based excited state and thus the poor fluorescence quantum yield ($\Phi = 0.0024$) for L. Such examples for related azine/imine compounds are very limited.[10] Further, literature reports reveal that the ring nitrogen atom (N^1) of the quinoline moiety is strongly hydrogen bonded with water molecules in aqueous or mixed aqueous medium and forms a stoichiometric complex between excited state quinoline and a solvent molecule (exciplex), which resulted in the appearance of a weaker emission band at longer wavelength.[11] Concurring to this report, the emission intensity for L at 406 nm was found to be enhanced with a systematic increase in the proportion of water from 16% to 40% in the THF/H$_2$O medium (Figure 2a). It has been argued that H-bonding interferes with the
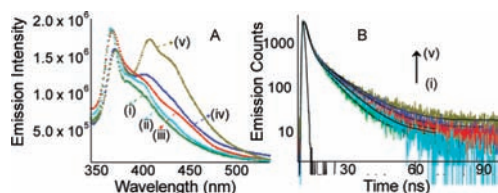
Figure 2. (A) Changes in fluorescence (λ_{exc}, 330 nm; slit width, 5/5 nm) and (B) time-resolved emission spectra (λ_{exc}, 360 nm LED; λ_{mon}, 425 nm) of L with increasing water/THF ratio from (i) 0%, (ii) 16%, (iii) 20%, (iv) 33%, to (v) 40%.

internal conversion and/or intersystem crossing of the excited singlet state, and this could account for the observed increase in emission intensity at 406 nm for L with an increase in [H$_2$O] in the medium.[11] This is also supported by the results obtained from the Time Correlated Single Photon Counting (TCSPC) studies (Figure 2b). As reported for the quinoline derivative, contribution of the longer-lived component of the biexponential decay rate constants for L ($\tau_1 = 1.6$ ns, 55%, $\tau_2 = 7.5$ ns, 45% in THF) was found to increase ($\tau_1 = 1.77$

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ns, 48%, $\tau_2 = 11.17$ ns, 52% in THF/H$_2$O (6:4, v/v) with increase in the [H$_2$O].

The possibility of a photoinduced electron transfer (PET) process involving the lone pairs of electrons on N^1 and N^2 of L and the excited fluorophore could also contribute,[12] along with the C=N isomerization, to the observed weaker emission.

In agreement with our observation for electronic spectra, a substantial enhancement in fluorescence intensity for L with a new band maximum at 420 nm appeared in the presence of added Hg^{2+}, while no change in emission spectra for L was registered when all other alkali/alkaline earth and common transition metal ions (Li$^+$, Na$^+$, K$^+$, Cs$^+$, Ca^{2+}, Mg^{2+}, Sr^{2+}, Ba^{2+}, Cr^{3+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}) were added (Figure 3A). In general, the Hg^{2+} ion is known
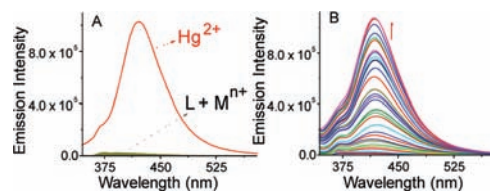
Figure 3. Fluorescence (A) scanning and (B) titration spectra (λ_{exc}: 330 nm, slit width 2/2 nm) of L (4.0×10^{-5} M) in THF/H$_2$O (6:4, v/v) with varying [Hg^{2+}].

to favor quenching of the luminescence of a fluorophore, to which it is covalently attached, through an efficient spin−orbit coupling.[6] However, in the present situation, the appearance of the new charge transfer emission band at 420 nm in the presence of added Hg^{2+} could be ascribed to the restricted C=N isomerization process of L on coordination to Hg^{2+} (Scheme 1). Restricted PET on binding to Hg^{2+} could also

Scheme 1. (A) C=N Isomerization in L and Its Restricted Rotation in $[\{(Hg^{2+})_2\}L](ClO_4)_4]$ and (B) X-ray Structure for HgCl$_2$ Bound to L
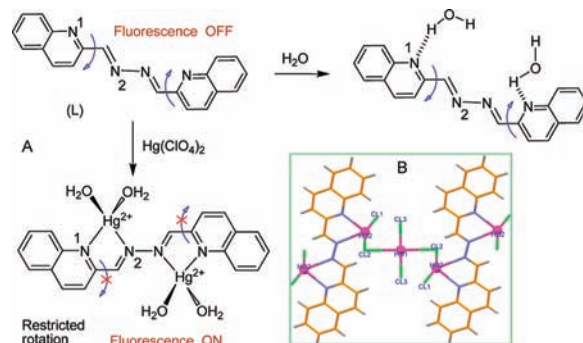
contribute favorably to this apparent *switch on* luminescence response. Thus, the inhibition of two non-radiative processes for L, bound to the Hg^{2+} ion, account for the substantial increase in overall fluorescence quantum yield of L ($\Phi^L = 0.0024$ to $\Phi^{\{(Hg_2^+)_2L\}} = 0.23$ with respect to quinine sulfate

in 0.1 M H_2SO_4). Results of the systematic fluorescence titrations also favor the 2:1 (Hg^{2+}:L) binding with binding constant of 9.0×10^6 M^{-2} L^2 in neutral water (1.20×10^7 M^{-2} L^2 in phosphate buffer of pH 7.2), which is close to the one evaluated from spectrophotometric titrations. Reversibility of the binding of Hg^{2+} to L was tested by adding KI solution. The I^- ion is well-known to bind strongly to the Hg^{2+} ion.[13] On addition of excess of KI to the solution of L·Hg^{2+} the fluorescence spectra for L was restored (Supporting Information). Thus, spectroscopic data suggest that this newly synthesized spectroscopic marker L could be used as a specific and efficient sensor molecule for Hg^{2+} in aqueous environment. The lower detection limit for Hg^{2+}, using this chemosensor (L), was also evaluated. The fluorescence studies reveal that L (20 μM) could be used for detection of the Hg^{2+} ion present in a sub parts per million level (0.4 ppm). Please note that the signal-to-noise ratio for this specific concentration was at least three.

However, single-crystal[14] analysis of L, coordinated to $HgCl_2$, showed different coordination geometry and stoichiometry than those that were evaluated from different spectral analyses (i.e., 2:1 for Hg^{2+}:L) using the $Hg(ClO_4)_2$ salt. This could be explained based on the much higher covalent nature of the Cl^- salts of Hg(II). As shown in Scheme 1, quinoline and imine nitrogen atoms (N^1/N^2) are bound to each Hg^{2+} (2) through the formation of a five-membered chelate ring with a distorted tetrahedral coordination geometry around each Hg(2) center with tetrahedral angles ranging from 67.1(3)° to 156.4(3)°. It is interesting to note that the coordination geometry around Hg(1) is distorted square planar with bond angle varying within the range of 87.85(12)° to 92.15(12)° in which two Cl(2) are involved in a weaker μ-Cl coordinate on (Hg1···Cl2 = 2.900(3)) and act as a bridge between the different Hg, maintaining the stoichiometry of the metal salt to ligand 3:1. However, it is worth mentioning here that the stoichiometry in solid state could be different from that observed in solution,[15] as the salt used and the conditions at which the X-ray single crystals grown were different from those in solution, used for recognition studies.

We explored the possibility of using L for the recognition of the Hg^{2+} ion in living cells (Epithelial cell line KB 31 cells) using confocal imaging experiments. Epithelial cell line KB 31 cells (EACC, Porton Down, Wilts, UK) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Carlsbad, CA), supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% antibiotic solution (Gibco).

One day before imaging, cells were trypsinized and plated on glass coverslips (Falcon BD Biosciences, San Josè, CA). The cultured cells were exposed to 40 μM $Hg(NO_3)_2$·H_2O in DMEM for 15 min at 37 °C, washed with PBS to remove the remaining mercury ions, and incubated with 10 μM L in DMEM for 30 min at 37 °C. Confocal imaging experiments of live cells were performed with a Leica TCS SP5 microscope (Leica Microsystems, Mannheim, Germany) with excitation at 380 nm and emission at 420 nm. Cells were immersed in PBS during the imaging process. Confocal microscopy images of live KB 31 cells loaded with 10 μM L for up to 1 h at 37 °C showed negligible intracellular fluorescence (Figure 4b). In contrast, cells treated with mercury and the compound L showed greatly enhanced intracellular fluorescence (Figure 4c). Thus, these results
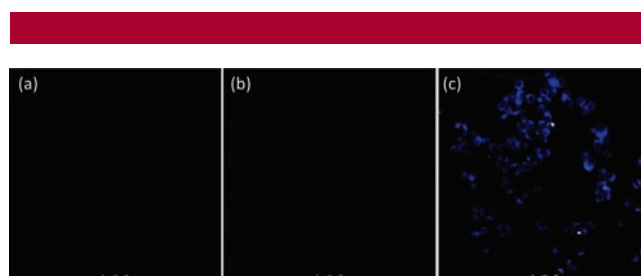
Figure 4. Confocal microscopic images of (a) KB 31 cells (b) loaded with 10 μM L and (c) 40 μM Hg^{2+} and 10 μM L.

demonstrate that L can be used to detect the Hg^{2+} ion present in the biological samples.

In summary, we synthesized a simple chemosensor that selectively binds the Hg^{2+} ion in aqueous media. We exploited two signal transaction mechanisms, namely, chelation-enhanced fluorescence and C═N isomerization, to report *fluorescence trun-on* response on binding of L to the Hg^{2+} ion. To the best of our knowledge, this is the first report where C═N isomerization, as a signal transaction mechanism, has been exploited for the recognition of the Hg^{2+} ion. We have provided crytallographic evidence for the binding mode of the bisazine moiety for Hg^{2+}. Confocal scanning microscope experiments revealed that L can be used to detect Hg^{2+} present in the KB 31 cells through an increase in intracellular fluorescence.

Supporting Information Available: Synthetic details, characterization data for the compound L, and selected spectroscopic data of L. This material is available free of charge via the Internet at http://pubs.acs.org.

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