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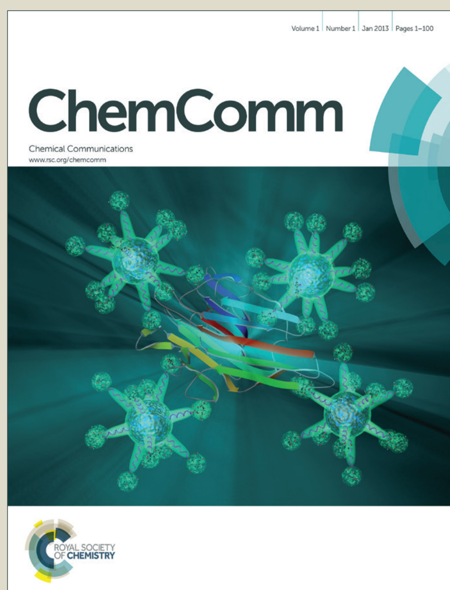

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Specific probe for Hg^{2+} to delineate even H^+ in pure aqueous buffer / Hct116 colon cancer cells: Hg(II)-η^2-arene π-interaction and a TBET-based fluorescence response

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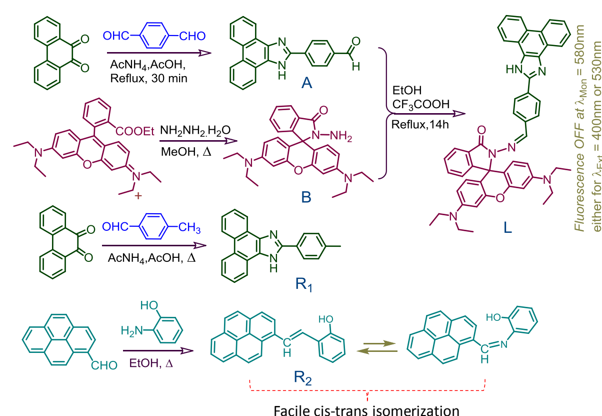
A new phenanthroimidazole-rhodamine conjugate constitutes a unique example of the role of a secondary bond in achieving a distinct TBET process induced by the Hg(II)-η^2-arene π-interaction in pure aqueous medium with a large pseudo-Stokes' shift of 200 nm. This molecule could also be used as an imaging reagent for detection of the cellular uptake of Hg^{2+} as low as 0.2 ppb in Hct 116 colon cancer cells.

Intramolecular triplet-triplet energy transfer is being pursued more recently for developing efficient fluorescence based receptors, as the average lifetime of the triplet state is much longer than the singlet state.[1] However, such triplet-triplet energy transfer is spin-forbidden by the dipole-dipole mechanism (Förster-type mechanism) and is only allowed by the electron exchange mechanism (Dexter-type mechanism).[1] Such triplet-triplet energy transfer requires either effective orbital overlap or coupling mediated by an appropriate conduit. More importantly, such through bond energy transfer (TBET) process is not limited by the fact that donor and acceptor fragments ought to have a spectral overlap. This offers the opportunity to achieve a large pseudo-Stokes shift to avoid self-quenching of the donor fluorophore and fluorescence detection errors because of excitation backscattering effects.[2] Additionally such energy transfer dyad also helps in avoiding the problem for photo-bleaching of the probe fluorophore.[2,3] For fluorophore dyad systems that have an insignificant spectral overlap between the donor emission and acceptor absorption, FRET process could be operational along with a TBET process. The challenge of demonstrating the TBET process in a small molecule fluorophore dyad without complete absence of spectral overlap has not been met till date, barring one recent example.[4]
Among various fluorophores, acyclic xanthene form of different rhodamine derivatives are being widely used as an imaging reagent for their high emission quantum yield, cell membrane permeability, non-toxic nature towards live cells and finally the *switch on* fluorescence response on conversion of a cyclic lactam form to the acyclic one.[5] However, such reagents generally have a strokes shift of about 50 nm and respond to H^+ as well as certain metal ion(s) with a fluorescence on response.[5] More recently, it is demonstrated that with appropriate design of a dyad that exhibited interrupted PET coupled TBET-based response not only to achieve a much larger strokes shift but also to distinguish the responses towards H^+ and a specific metal ion (Hg^{2+}) through appropriate choice of the excitation wavelength.[6] However, there existed a small spectral overlap between the donor and the acceptor fragments and this did not completely exclude the possibility of the FRET-based response along with the predominant TBET process. Despite several advantages, example of appropriate fluorescent probes based on TBET cassettes for intracellular imaging applications are actually rare.[3,6]



Scheme 1. Methodologies that were adopted for synthesis of **A**, **B**, **R_1**, **R_2** and **L**.

Among various metal ions, Hg^{2+} is one of the most potent neurotoxin known and its deleterious influences on human as well as on plant physiology are well documented in a range of literature reports.[7] Its presence, even at very low concentrations, leads to serious health hazzords.[7a] According to the international regulatory board (EPA, USA), the lowest Hg(II) concentration that is allowed for the safe drinking water is 0.2 ppb.[8] Keeping this in mind, it is imperative to develop a reagent that could specifically recognize, detect and quantitatively estimate mercury ion concentration as low as the above referred limit in pure aqueous/buffer medium having physiological pH. Among various options, fluorescence-based probe molecules are preferred as this offer the possibility of using such reagent as a sensitive imaging reagent for detection of cellular uptake of Hg^{2+}.[5,9] Hg^{2+} is known to be an efficient quencher for molecular fluorescence due to a facile spin-orbit coupling process.[10] The solvation enthalpy for Hg^{2+} is also significant (1824 kJ $mole^{-1}$). Thus, designing of an ultrasensitive fluorescence-based specific probe for Hg (II) with high Strokes shift is a challenging issue.
In the present communication, we have described a new probe molecule that has allowed us to achieve the specificity in binding to Hg^{2+} in aqueous buffer medium through a Hg (II)-η^2-arene complex formation. This not only allowed us to delineate Hg^{2+} from H^+ based on a distinctly different fluorescence response, but also helped us to achieve a significant pseudo Stokes shift of ~200 nm through a TBET-based

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response. Considering the fact that most spirocyclic rhodamine derivatives in their lactam form are reactive towards H^+ and show similar *fluorescence on* response at pH < 4.0 as these shows on binding to a specific cationic analyte, the present reagent (**L**) is unique. Different photo induced processes are operational on binding to Hg^{2+} and H^+. Such example is rare in the contemporary literature.

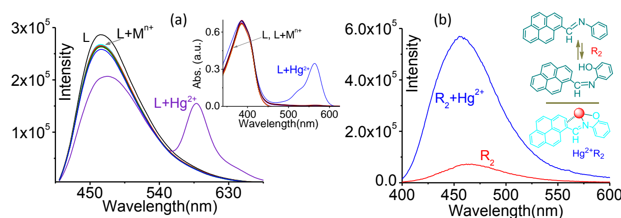

Fig 1. Changes in (a) Emission (λ_{Ext} of 400 nm; slit = 1/1 nm) and (Inset) absorption spectra of the receptor **L** (20 μM) in absence and presence of different metal ions (M^{n+} = Li^+, Na^+, K^+, Mg^{2+}, Ca^{2+}, Ba^{2+}, Sr^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+}, Cd^{2+}, Co^{2+}, Fe^{2+}, Fe^{3+}, Cr^{3+}, Pd^{2+}; and Pb^{2+}); (b) Emission spectra of $\mathbf{R_2}$ (20 μM) and $\mathbf{R_2}$ in presence of Hg^{2+} (40 eq) by using λ_{Ext} = 380 nm and (Inset) the scheme showing the restriction in *cis-trans* isomerisation of $\mathbf{R_2}$ on binding to Hg^{2+}. All studies were performed in aq. HEPES buffer (10 mM; pH 7.2) solution having 0.4 mM TX100.

Procedures followed for synthesis of **L** and model reagents $\mathbf{R_1}$/$\mathbf{R_2}$ are shown in scheme 1. Detailed synthesis of **L**, $\mathbf{R_1}$ and $\mathbf{R_2}$, their spectral (^{1}HNMR, ^{13}C NMR,) and analytical characterization data are provided in the supporting information.† All such data confirmed that **L**, $\mathbf{R_1}$ and $\mathbf{R_2}$ were isolated in desired purity. Reagent **L** showed an intense absorption band at 386 nm for the benzimidazole derivative of the phenantherene moiety and beyond this, no other absorption band was observed when spectra were recorded for 20 μM solution of **L** in aq. buffer medium (aq. HEPES buffer; 10 mM; pH 7.2) containing 0.4 mM Titron X100 (TX100) (Fig 1a(Inset)). This assignment was based on the observed electronic spectral band for the model compound $\mathbf{R_1}$ at 375 nm. Biologically benign neutral surfactant (TX100), allowed the reagent **L** to get trapped inside its micellar structure and to solubilize in pure aqueous buffer medium and such solution was used for all studies unless mentioned otherwise.

Following excitation at 386 or 400 nm, an intense and broad emission band with maximum at 464 nm was observed and this was attributed to the imidazole-based emission of **L**. This was further confirmed by comparing this emission spectra with that for the model reagent $\mathbf{R_1}$. As anticipated, no rhodamine-based emission band could be detected owing to its spirocyclic lactam structure (Figure 1a).[5] Emission spectra for **L** (20 μM) were recorded (λ_{Ext} of 400 nm) at different pH and results of such studies revealed that the spirocyclic form of **L** was stable for the pH range 5-10. On further lowering of the solution pH (below 4.0), a significant lowering of the imidazole based emission band at 464 nm was observed along with a barely detectable emission intensity enhancement at around 587 nm.†

However, excitation of the similar solution with 530 nm showed an intense emission band at 587 nm.† These results revealed two important aspects: Firstly, acyclic xanthene form was produced in solution having pH < 4.0;[5] however, the absence of any absorption below 420 nm for the acyclic xanthene form failed to give any sensible emission band beyond 530 nm for λ_{Ext} of 400 nm.[5] Secondly, absence of any emission band beyond 500 nm for the model reagent $\mathbf{R_1}$ also excluded the possibility of any FRET process with benzimidazole as donor and rhodamine moiety as acceptor.

Inset of Fig 1 clearly reveals that among various metal ions, interaction of the reagent **L** with Hg^{2+} is strong enough to induce the conversion of cyclic lactam form to the acyclic xanthene form with subsequent appearance of a new absorption bands at 560 nm. Emission spectra recorded for such a solution showed an emission bands at 464 and 587 nm (λ_{Ext} of 400 nm) Fig 1a. Relative emission quantum yield for Hg^{2+}.**L** (Φ =0.52) at 464 nm was less as compared to that was observed for free **L** (Φ =0.78). B-H. plots, obtained by using data available from systematic absorption and emission spectral titrations at 25°C, revealed association constant of (2.9 ± 0.5) x 10^3 M^{-1} and (2.6 ± 0.5) x 10^3 M^{-1}, respectively. Binding stoichiometry of 1:1 was ascertained from the good 'linear fit of the B.H plot and the ESI-Ms data.† The new emission band at 587 nm was attributed to the formation of Hg^{2+}.**L** and the acyclic xanthene form. As anticipated, higher association constant of (8.2 ± 0.5) x 10^4 M^{-1} was evaluated from luminescence titration in acetonitrile medium, while the binding stoichiometry remained same. Fluorescence response at around 464 nm is discussed in details following the discussion on the binding mode of the reagent **L** in Hg^{2+}.**L**.

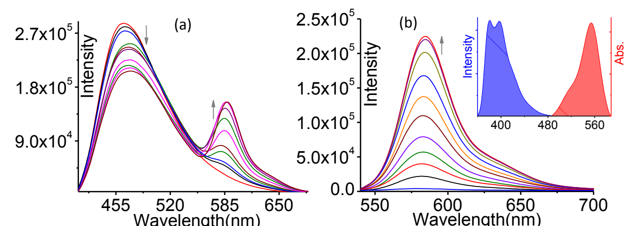

Fig 2. Change in luminescence spectra for **L** (20 μM) in presence of varying [Hg^{2+}] (0 - 30 equiv.) using (a) λ_{Ext} = 400 nm; (b) λ_{Ext} = 530 nm; (slit width 1/1 nm). Inset: Overlap spectra for emission and absorption spectrum of the donor ($\mathbf{R_1}$) and acceptor (acyclic xanthenes form of B), respectively. All studies were performed in aq. HEPES buffer (10 mM; pH 7.2) solution having 0.4 mM TX100.

To have a better insight about the possible binding mode for Hg^{2+} to **L**, detailed NMR studies were performed in d_6-DMSO. Distinct downfield shifts were observed for all protons and this shift was more significant for -HC=N. Such shifts are anticipated for H_{imine}, where N_{imine} is involved in coordination to a metal ion.[11a,b] Relatively higher downfield shift (Δδ = 0.23 ppm) for the proton indicated with "•H" (Scheme 1), compared to other aryl protons, suggested a Hg(II)-η^2-arene π-interaction (ESI). Such an Hg(II)-η^2-arene π-interaction is not a very uncommon in contemporary litera-ture.[5a,6] Also, ^{13}C NMR spectra revealed that the signal for the tertiary C-atom for the spirocyclic rhodamine moiety for **L** at 66.83 ppm disappeared when **L** was reacted with Hg^{2+}. This signified the formation of acyclic xanthenes form. Based on these NMR and other spectral (electronic, luminescence and ESI-Ms) observations, proposed binding mode of the acyclic xanthenes form of **L** to Hg^{2+} involving a Hg(II)-η^2-arene π-interaction is shown in the Scheme 2. Further support to such a proposition was achieved from the results of the ^{1}H NMR spectral studies with the analogous model compound $\mathbf{R_2}$ in absence and presence of Hg(II) in CD_3CN, where a distinct downfield d (Δδ = 0.32 ppm) shift was observed for the aryl proton (H^*) in $\mathbf{R_2}$ on binding to Hg^{2+}. This Hg (II)-η^2-arene π-interaction would restrict the *cis-trans* isomerization across the HC=N functionality in **L** or $\mathbf{R_2}$.

To understand the fluorescence responses of **L** on binding to Hg^{2+}, luminescence property of the model compound $\mathbf{R_2}$ was examined. Reagent $\mathbf{R_2}$ showed low emission quantum yield (ΦR_2= 0.009) when excited at 380 nm (Figure 1b & Scheme 1), the λ_{Max}^{Abs} for the pyrene based transition. This low $\Phi_{0.009}$ value was attributed to a fast non-radiative deactivation of the pyrene based excited state due to a rapid *cis-trans* isomerization process.[11] However, on binding to Hg^{2+}, a significant enhancement in its emission quantum yield was observed due to the restricted *cis-trans* isomerization (Figure 1b & Scheme 2). There are literature reports, which support such a proposition.[5f,11]

Binding of Hg^{2+} to **L** would also impose such restriction on the *cis-trans* isomerization of the benzimidazole derivative of the phenanthrene fragment and was expected to enhance the quantum yield for the emission spectral band having maximum at 464 nm. Interestingly, such enhancement was not observed when solution of **L** was excited at 400 nm and on the contrary a decrease in the emission intensity at 464 nm was observed with the onset of a new emission band with maxima at 587 nm on binding to Hg^{2+}. Further, Φ (λ_{Ext} = 400 nm, λ_{Max}^{Ems} of 587 nm) for Hg^{2+}**B** (Scheme 2) was evaluated as 0.025, while that for Hg^{2+}.**L** under identical experimental conditions was 0.54. These data clearly indicate that an efficient energy transfer process is operational from the donor Hg^{2+}-bound phenanthroimidazole moiety to the acceptor Hg^{2+}-

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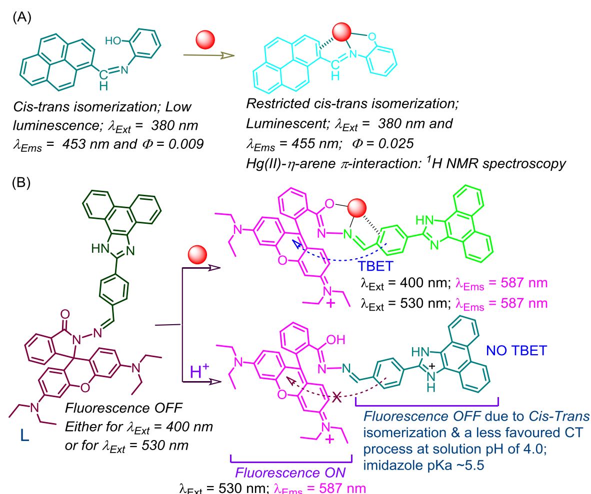
bound rhodamine fragment in Hg^{2+}.**L**. In absence of any possibility of the FRET process, all these collectively suggested the possibility of an efficient through bond energy transfer (TBET) from the benzimidazole derivative of the phenanthrene fragment (donor)[12] to the acyclic xanthene form of the rhodamine moiety (acceptor). Efficiency of the TBET process was evaluated as 87%.[2e,6,13a]



Scheme 2. (B) Proposed mode of binding in $Hg^{2+}.R_2$ and Hg^{2+}.**L** along with the presumed energy transfer pathway.

For the efficient TBET process on binding to Hg^{2+}, one would anticipate a significant and ratiometric decrease in the emission intensity at λ_{Max} of 464 nm, which was not observed in the present study. This apparent anomaly could be explained if we consider the fact that the restricted *cis-trans* isomerization (involving the benzimidazole moiety in Hg^{2+}.**L**) was actually operational and would account for fluorescence enhancement at ~464 nm. This presumption was amply supported by the observed fluorescence enhancement (*vide supra*) of the model compound $\mathbf{R_2}$ (Fig. 1b). Thus, these two opposing influences on emission intensity at 464 nm resulted only a partial decrease in emission intensity at ~ 474 nm and did not allow us to achieve the ratiometric fluorescence response which otherwise was anticipated for a TBET based luminescence responses.

Various TBET cassettes that are reported in the literature, including the one reported by one of the research groups authoring this article,[6a] have certain amount of spectral overlap between donor emission and acceptor absorption, which do not completely exclude the possibility of certain amount of FRET process along with the TBET process. To the best of our knowledge this is the first report that describes the donor emission has no spectral overlap with the acceptor absorption (Fig 2 Inset).

A closer look at the steady state emission data also reveal that excitation of the benzimidazole derivative of the phenanthrene moiety in **L** (λ_{Ext} of 400 nm) showed intense emission band with maximum at ~ 587 nm on binding to Hg^{2+} in aq. buffer medium having pH 7.2, but no such emission band was observed when analogous spectra were recorded at pH < 4.0 (Scheme 2B). Thus, this offered us the opportunity to use a rhodamine-based reagent to distinguish Hg^{2+} from H^+ based on the vastly different luminescence responses, which is not common for rhodamines based receptors.[4] Further, the difference of 200 nm between the donor absorption (387 nm) and the acceptor emission (587 nm) for Hg^{2+}.**L** was comparable to the highest value for the Stokes shifts (202 nm) reported earlier for another TBET based cassette.[3b] Further, steady state emission spectra recorded for a physical mixture of equimolar amount of two model reagents, $\mathbf{R_1}$ and Hg^{2+}.**B**, revealed no detectable quenching or enhancement of luminescence of $\mathbf{R_1}$ and Hg^{2+}.**B**, respectively, when λ_{Ext} of 375 nm (λ_{Max}^{Abs} for $\mathbf{R_1}$) was used. These excluded the possibility of any inter molecular energy transfer process,[4] which further corroborated an efficient TBET process. The lowest detection limit (3σ/slope)[13b] was evaluated and it was found to be as low as 0.48 ppb for Hg^{2+}. The reversibility of the binding process between **L** and Hg^{2+} was also established. On addition of aq. solution of $EDTA^{2-}$ to an aq. HEPES buffer solution (20 μM) of Hg^{2+}.**L** (pH = 7.2), the original spectrum for the spirolactam form of **L** was restored. Higher binding affinity of Hg^{2+} towards $EDTA^{2-}$ led to the formation of Hg^{2+}-$EDTA^{2-}$ and regeneration of **L**.

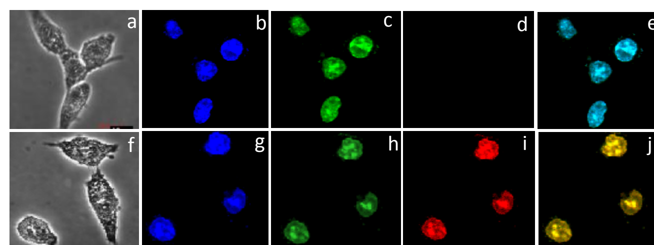


Fig 3. Confocal images of Hct116 cells treated with **L** (10 μM): (a, f) Bright filed images of Hct116 cells as control; (b) and (g) Co-staining of **L** with nuclear staining dye DAPI (DAPI: 4′,6-diamidino-2-phenylindole) from blue channel, (c - e) cells incubated with only **L** (10 μM): (c) at green channel, (d) at red channel and (e) overlay images of (b) and (c); (h - j) cells incubated with **L** (10 μM) for 30 min and then with then exposed to Hg^{2+} (4 ppb) for 30 min at 37°C; (h) at green channel, (i) at red channel, (h) overlay image of (h) and (i); For all studies, a laser source of λ_{Ext} of 400 nm was used.

MTT assay was performed to evaluate the toxicity of this reagent towards Hct116 cells.[13b] After ascertaining the fact that the reagent **L** showed only moderate toxicity towards live cancer Hct116 cells, possibility of using this reagent for detection of Hg^{2+} uptake in live cancer Hct116 cells were explored using a laser excitation source of 400 nm. Initially, live Hct116 cells were incubated with only **L** (10 μM) for 30 min at 37° C. After necessary and through washing, these cells showed intense fluorescence in the green channel due the luminescence from the benzimidazole derivative of the phenanthrene moiety (Figs. 3c). Further, confocal laser microscopic images of the Hct116 cells that were pre-treated with 10 μM of the reagent **L** (and thoroughly washed for removal of surface adhered reagent) and after follow-up treatment with Hg^{2+} (4 ppb) caused a distinct decrease in observed fluorescence intensity in the green channel with an onset of a strong fluorescence in the red channel (Figs. 3h & 3i). These demonstrated two important aspects: reagent **L** was cell membrane permeable and could be used as an imaging reagent for the detection of Hg^{2+} uptake in living cell. Additional studies confirmed that the uptake of Hg^{2+} as low as 0.2 ppb, in live Hct116 cells could even be detected in confocal images (ESI Fig. 18), which is safe limit for Hg^{2+} ion concentration (set by the U.S. EPA) for the environmental primary standard.[8]

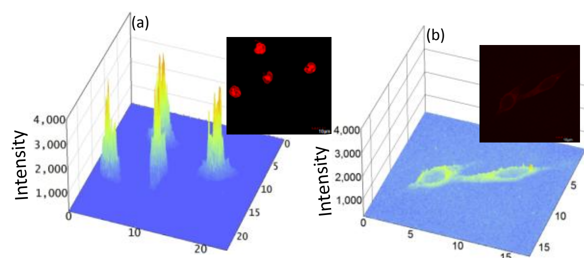


Fig 4. 3D Confocal images of Hct116 cells (pre-treated with **L** (10 μM)) on exposure to a solution having (a) Hg^{2+} (4 ppb) in aq. HEPES buffer (pH 7.2) and (b) pH 4.0 (aq. HEPES buffer) for 30 min at 37°C (λ_{Ext} = 400 nm) at red channel.

Further, in order to confirm the ability of the reagent **L** to distinguish the cellular pH ≤ 4.0 and cellular Hg^{2+} ion uptake, live Hct116 cells were pre-incubated with buffer solution having pH ~ 4.0 as well as Hg^{2+} solution (pH 7.2) and respective solutions were subsequently exposed to a solution of **L**. Confocal images of these two sets of cells were recorded after excitation at 400 nm (excitation of the imidazolium derivative) and observed confocal images were distinctly different (Fig. 4). As anticipated for cells exposed to solution having pH ~ 4.0, TBET process was not operational and only insignificant intracellular emission was observed at red channel; while due to an efficient TBET process, a

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strong intracellular emission were observed at red channels for cells that were pre-treated with Hg^{2+}. Thus, observed results allowed to distinguish the responses of the reagent towards H^+ and Hg^{2+}. Interestingly, a close examination of the images shown in Fig 4 also revealed that reagent **L** could detect Hg^{2+} ion uptake in the nucleus of the Hct116; while that in solution having pH 4 could only stain the cytosol. Imidazole part of the receptor **L** has pKa around 5.5 and predominant LH^+ (Scheme 2b) form is expected to exist at pH ≤4.0.[14a] Confocal image shown in Fig.4b clearly revealed that $\mathbf{LH^+}$ became nuclear membrane impermeable. Presumably, protonation of **L** at low pH was expected to lead to an increase in its lipophilicity, which adversely affected the uptake of the reagent **L** in the nucleus. Such a proposition is put forward recently by Friberg et. al..[14b] Such an example for rhodamine based reagents is rare in the contemporary literature.

Conclusions

In conclusion, we have synthesized phenanthroimidazole appended rhodamine based fluorescent probe **L** which showed an efficient TBET based fluorescence response on specific binding to Hg^{2+} in an ensemble of several other cations. Appropriate design of the receptor molecule **L** and the Hg(II)-η^2-arene π-interaction allowed us to exploit the role of the secondary bond in achieving the TBET process with a pseudo Stokes shift of 200 nm. Interestingly, unlike most other rhodamine based receptors, this reagent showed distinctly different fluorescence-based output signal on binding to H^+ in solution having pH ≤ 4. Use of 0.4 mM TX100 in aqueous HEPES buffer solution (10 mM; pH 7.2) allowed the reagent **L** to be trapped inside the micellar structure of TX100 and this allowed all recognition, detection and binding studies to be performed in pure aqueous medium having physiologically relevant pH of 7.2. This reagent was found to be cell membrane permeable and could be used as a fluorescent probe for imaging Hg^{2+} ion uptake in live Hct116 colon cancer cell lines.

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Notes and References

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Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/c000000x/

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