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ARTICLE TYPE

FRET based '*red-switch*' for Al^{3+} over ESIPT based '*green-switch*' for Zn^{2+}: Dual channel detection with live-cell imaging on a dyed platform

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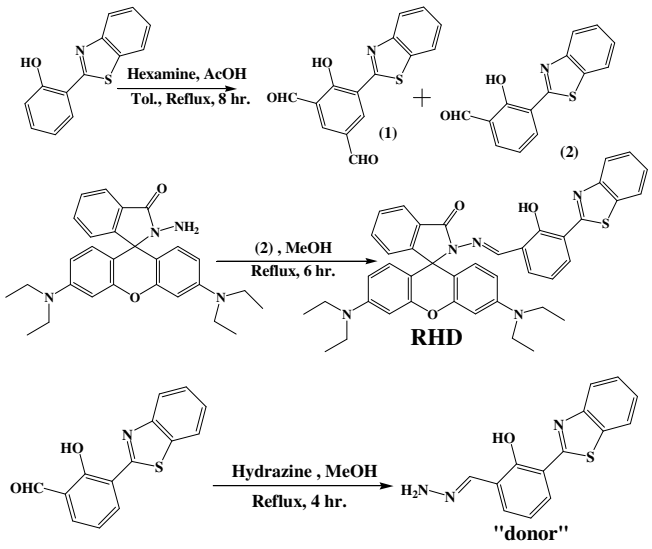
Our designed chemosensor, Rhodamine-HBT-dyed (RHD), selectively detects two biologically important ions (Al^{3+} and Zn^{2+}) at two different wavelengths ('naked-eye' colors red and green, respectively) through two different mechanisms (*i.e.* FRET and ESIPT) in ppm level. The sensor could also detect Al^{3+} ion through the displacement of Zn^{2+} ion in vivo.

Fluorescence (or Forster) resonance energy transfer (FRET), a non-radiative procedure, is a result of a proper arrangement of energy-donor-acceptor architecture of fluorosent molecules in its excited-state. Influenced by the scope of spectral overlap between donor emission with acceptor absorption and the distance among donor and acceptor, excited donor transfers energy to an acceptor without photoemission.[1] In the area of chemosensor, FRET containing scaffold has its potentially realistic benefits in cell physiology and optical therapy, as well as selective and sensitive sensing in the direction of targeted molecules or ions.[2]

On the other hand, due to their central properties like ultra-fast reaction rate and tremendously huge fluorescence Stokes shift,[3] ESIPT (Exited state intramoleculer proton transfer) compounds have also drawn a lot of significance. In ESIPT, the enol isomer, which is lower in energy than the keto isomer in the electronic ground state, undergoes the proton transfer reaction upon excitation to the excited state. Upon irradiation, 2-(2-hydroxyphenyl)benzothiazole (HBT) and its derivatives, generate the excited-state intramolecular proton transfer (ESIPT) tautomers (the keto forms), which show fluorescence more strongly at longer wavelength compared to the phenol forms. Many researchers also intended anion and cation sensors with ESIPT mechanism, taking (HBT) as a model moiety for this purpose.[4] We also took well explored HBT as an ESIPT moiety with a conjugated rhodamine system to take the advantage of the FRET. The rhodamine fluorophore exhibits a longer wavelength emission (over 550 nm) which results from its particular structural properties that help avoiding the influence of background fluorescence (below 500 nm).[5] Thus, the ESIPT containing Rhodamine-HBT Dyad (RHD) platform is a unique one showing various exciting properties. This unique small molecule fluorescent dye, *i.e.* RHD, becomes important when Al^{3+}, the most abundant (8.3% by weight) metallic component (the third most rich of all elements following oxygen and silicon) and Zn^{2+} (the next most abundant transition metal ion after iron in the human body) are selectively detected through a different channel by this receptor in vitro and in vivo. Many symptoms of aluminium toxicity mimic those of Alzheimer's disease, osteoporosis, colic, rickets, gastrointestinal problems, interference with the metabolism of calcium, extreme nervousness, anemia, headaches, decreased liver and kidney function, memory loss, speech problems, softening of the bones and aching muscles.[6] In addition, Zn (II) is a very important ion species in many biological activities such as neural signal transmission, gene expression, cellular metabolism and DNA binding or recognition.[7] Thus, in vivo detection of Al^{3+} and Zn^{2+} is very important from the view of human health.

Though few fluorescent sensors specific for aluminium [8] and both aluminium and zinc ions in one stand have been reported,[9,10] there are many aspects left to be improved. More importantly, it is still a challenge to develop dual channel fluorescent sensors for two different biologically important ions that can develop water-soluble and cell permeable devices combining FRET and ESIPT in a single dyed platform.

Scheme 1: Synthetic outline of RHD.



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The synthesis of the sensor *i.e.* RHD is shown in Scheme 1. When HBT is treated with hexamine (excess) and acetic acid in refluxing condition for 8 hours, it gives a mixture of the mono-aldehyde (**2**) and di-aldehyde (**1**) of HBT. Interestingly, in our earlier report[11] we can see that the major product is **1** after 21 hours. Finally, after separation, the desired product (**2**) is condensed with rhodamine hydrazide in methanol and it gives the desired receptor (RHD) [details of the synthetic procedure, NMR, mass and X-ray crystallographic data are given in Electronic Supporting Information† (ESI†)].

In FRET phenomenon, the overlap of the absorption spectrum of the acceptor with the emission spectrum of the donor is proportional to efficiency of energy transfer from donor to acceptor. The absorption spectrum of the open spirocycle form of the rhodamine B develops a significant overlap with the emission spectrum of donor and clearly offers the possibility of a FRET process (Fig. 1).

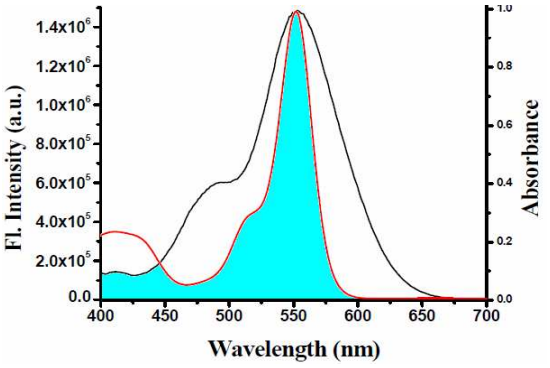

Figure 1: The overlap between the emission and absorption spectra of the donor ("donor") and acceptor moieties.

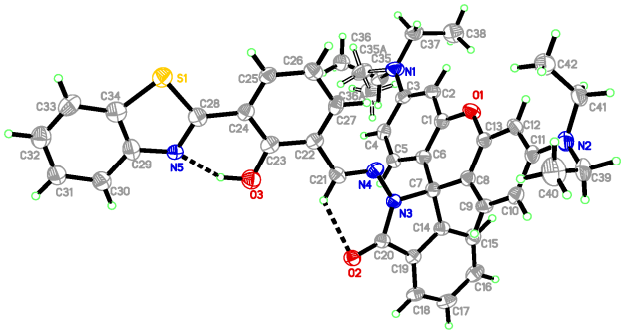

Figure 2: The ortep view of the receptor RHD in atom numbering scheme with 50% probability displacement ellipsoids for non-H atoms. Intramolecular hydrogen bonds and minor component of disorder are shown as dashed lines and open bonds, respectively.

In the structure of RHD, the xanthene ring system is approximately planar, with a maximum deviation of 0.045 (3) Å, and its mean plane makes dihedral angles of 75.92 (13), 78.15 (9) and 89.45 (9) ° with the benzene ring, benzothiazole and isoindoline ring systems, respectively. The benzene ring forms dihedral angles of 7.30 (13) and 3.68 (14)° with the mean planes of isoindoline (maximum deviation = 0.019 Å) and benzothiazole (maximum deviation = 0.034 Å) ring systems, respectively. The dihedral angle between isoindoline and benzothiazole ring systems is 19.65 (11)°. The molecular structure is stabilized by intramolecular O3—H1*O*3···N5 and C21—H21*A*···O2 hydrogen bonds (Table 2; ESI†), which generate S(6) ring motifs (Fig. 2).

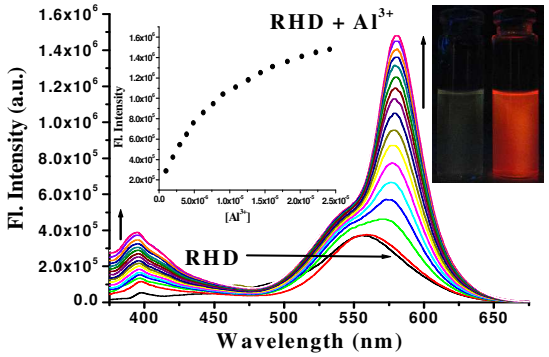

Figure 3: Fluorescence titration spectra of RHD (c = 2.0 x 10^{-5} M) in presence of Al^{3+} (c = 2.0 x 10^{-4} M) at pH 7.5 in $CH_3CN:H_2O$ = 1:1 (v/v) with the binding isotherm and visual color change of RHD in presence of Al^{3+} under uv-light.

In emission spectroscopy, the two peaks of RHD are centered at 398nm and 556nm (λ_{ex}= 365nm) confirming its typical ESIPT character i.e. the lower range intensity peak appeared for 'enol' and the higher range peak associated with 'keto' form. As shown in Figure 3, upon incremental addition of Al^{3+} solution, the higher wavelength peak is steadily red-shifted and finally saturated at 581nm (25nm red shift) with the addition of 3 equivalents of Al^{3+} with a huge intensity which help us to detect the particular metal ion with 'naked-eye' (pale yellow to deep red).

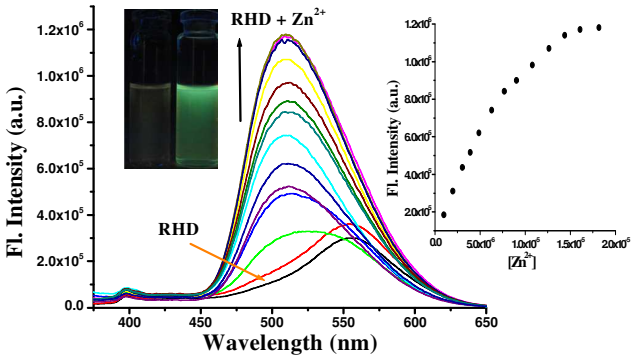

Figure 4: Fluorescence titration spectra of RHD (c = 2.0 x 10^{-5} M) in presence of Zn^{2+} (c = 2.0 x 10^{-4} M) at pH 7.5 in $CH_3CN:H_2O$ = 1:1 (v/v) with the binding isotherm and visual color change of RHD in presence of Zn^{2+} under uv-light.

On the other hand, the gradual increase of the peak at 398nm confirmed that the ESIPT process is interrupted through the association of Al^{3+} with RHD, probably due to its structural change on complexation. Thus the exitation energy is passed from HBT to the 'opened-up' rhodamine moiety, which is an

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essential condition for a successful FRET phenomenon. The association constant is found to be 5.22×10^4 M^{-1} using Benesi-Hildebrand equation (ESI†). Quantum yield of the receptor i.e. RHD is changed from 0.051 to 0.753 in presence of Al^{3+} (ESI†). With the addition of Zn^{2+} in RHD solution, the higher wavelength peak is shifted towards left and finally gets saturated at 509nm (i.e. 47nm blue shift).

The solution of RHD becomes green in 'naked-eye' with the addition of aqueous solution of only 2 equivalents of $ZnCl_2$ (Fig. 4) with an association constant 4.05×10^4 M^{-1} (ESI†).

From the spectral data, it is clear that due to the chelation of Zn^{2+} with RHD, the suppressed ESIPT (probably due to the formation of the hydrogen bond with the imine nitrogen) is recovered (quantum yield becomes 0.654 from 0.051) i.e. the particular proton is transferred freely in opened environment (Scheme 2) and we get a characteristic green fluorescence of HBT moiety. The calculated detection limits for Al^{3+} and Zn^{2+} are 0.76 µM and 0.32 µM, respectively, using the equation (Sb1* K)/S [ESI†]. Job plot suggests the 1:1 stoichiometry for both case.

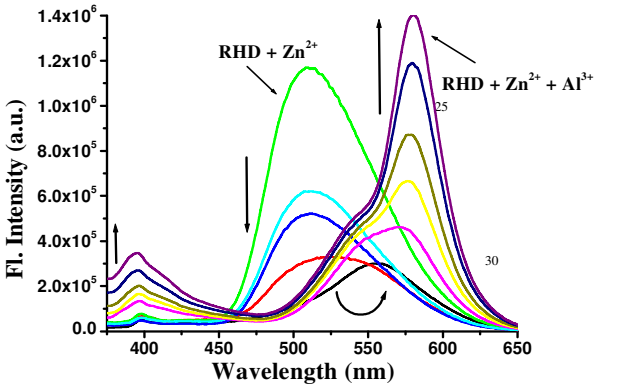

Figure 5: Fluorescence titration spectra of RHD-Zn ensemble ($c = 2.0 \times 10^{-5}$ M) in presence of Al^{3+}($c = 2.0 \times 10^{-4}$ M) at pH 7.5 in $CH_3CN:H_2O$ = 1:1 (v/v) .

In continuation of our previous report[10] and being influenced by other exciting previous work,[9,12] that deals with displacement approach, Al^{3+} is added to the greenish fluoroscent solution of RHD-Zn^{2+} ensemble. Interestingly, the intensity band centered at 509nm is gradually decreased and finally vanished with the addition of only 0.5 equivalent of Al^{3+} solution which confirmed the displacement of Zn^{2+} from RHD-Zn^{2+} ensemble. The new intensity band is raised and saturated at 581nm with addition of another 3 equivalents of Al^{3+} which concludes the formation of RHD-Al^{3+} complex (Fig. 5). In ^{1}H-NMR spectra, in presence of Zn^{2+} the enol proton of RHD is present but when Al^{3+} is added to the mixed solution the particular proton disappears, which also confirmed the displacement model shown in Scheme 2.

In order to determine the membrane permeability of the receptor (RHD) and its ability of binding to Al^{3+}/Zn^{2+} in living cells, HeLa cells were first treated with $ZnCl_2$ followed by the addition of RHD (30 min. + 30 min.). As shown in the Figure 6, the cells showed green fluorescence in FITC channel when they were treated with Zn^{2+} followed by the receptor. When further $Al(NO_3)_3.9H_2O$ is added to the previously Zn–treated HeLa cells (incubation for another 30 min.), we see a bright red color fluorescence in TRITC channel which confirmed the displacement of Zn^{2+} with Al^{3+} in vivo. In absence of Al^{3+} or Zn^{2+} , no fluorescence was observed in cells except DAPI, which is used for staining nuclei of the cells. The result evidently documented that the RHD could permeate the plasma membrane of the cells and give two dissimilar specific fluorescence in presence of Al(III) over Zn(II).

Scheme 2: Probable dual channel binding mode of Zn^{2+} and Al^{3+} towards RHD (supported by NMR and MS spectra ESI†).

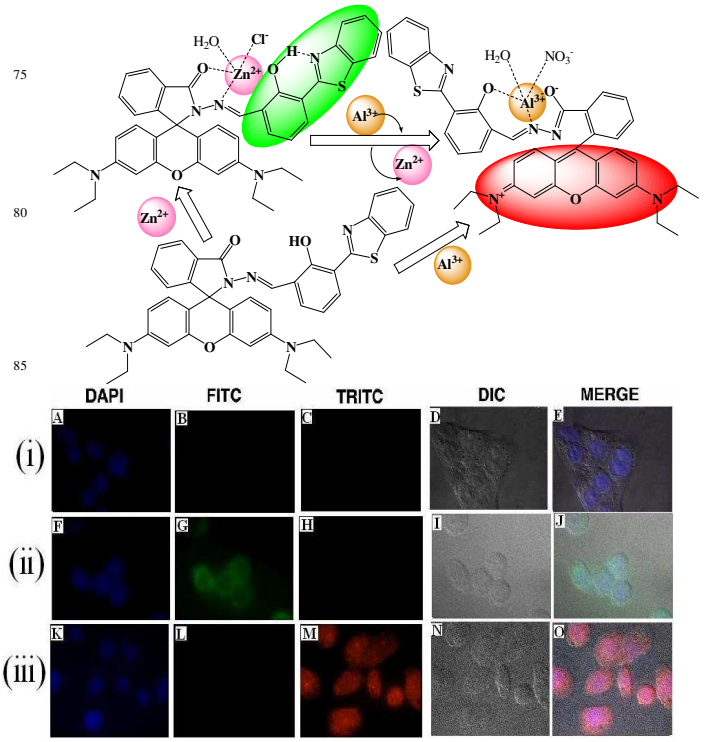

Figure 6: Fluorescence images of HeLa cells incubated with 50 µM of the Receptor (RHD) in absence (i), in presence of 50 µM of Zn^{2+} (ii) and in presence of 50 µM of Zn^{2+} then 50 µM of Al^{3+} (iii) with Corresponding differential interference contrast (DIC) and merge images of the cells.

Prompted by its high sensitivity in solution, the practical application of RHD in solid state was also investigated. Test strips (TLC plate sticks) were prepared to detect Al^{3+} with different concentrations. These test strips demonstrated apparent color changes under a UV lamp also (ESI†).

In summary, we have designed and synthesised a cell permeable small molecule fluorocent probe (RHD) for dual channel 'naked-eye' in vitro and in vivo detection of Al^{3+} and Zn^{2+} with FRET and ESIPT mechanism correspondingly with a lower detection limit. This procedure opens up new technique for the recognition of two biologically important cation in single stage. The sensor also showed an outstanding

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presentation towards solid phase (TLC plate) detection.

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Notes and references

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†Electronic Supplementary Information (ESI) available: [details of synthetic procedure, X-ray crystal structure (CCDC 947172) and spectral data are available]. See DOI: 10.1039/b000000x/

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