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A simple rhodamine-based dual signalling reversible molecular switch for recognition of Al(III) with Promising applications for advanced logic operations – 'OR', 'Keypad Lock' & 'INHIBIT' logic function and cell-imaging studies†.

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A simple salicylaldimine-based receptor equipped with rhodamine moiety (L^3) has been designed and synthesized for detection of Al^{3+} ions, which exhibits high sensitivity over other metal ions in aqueous buffer-methanol solution at physiological *p*H. It displays quick responses through visible colorimetric as well as fluorogenic changes on 1:1 binding to Al^{3+}, as delineated by absorption and fluorescence titrations and also by Job's method and ESI-MS^+ studies. However, it exhibits calorimetric but not fluorometric responses towards Cu^{2+} and hence limits its colorimetric application towards the detection of Al^{3+} in the presence of Cu^{2+} ions. The properties of the probe like (i) solubility in aqueous buffer-methanol, (ii) cell-permeability and (iii) non-toxic nature towards cell may provide an opportunity for the *in vitro*/ *in vivo* bio study. The detection limit of Al^{3+} calculated by the 3σ method was found to be 24.8 pM. It is also found to be useful in the construction of logic gates; namely '**OR'** gate (from absorption) and molecular '**keypad lock'** (from emission) by the two chemical inputs (Al^{3+} and Cu^{2+}) in the proper sequence of addition. The **keypad lock** operation is particularly important, as the output of the system depends not only on the proper combination but also on the order of input signals, creating the correct password that can be used to ''open'' this molecular keypad lock through strong fluorescence emission at 552 nm. Besides, "OFF–ON–OFF" fluorescence behavior observed in the presence of Al^{3+} and EDTA strengthens the potential applications of the L^3–Al^{3+} system as a device with '**INHIBIT**' logic gate functions. As a whole, its various logic gate properties may improve its impact for the development of new-generation '**intelligence**' digital devices. To the best of our knowledge, this is the first report on the fluorescence emission of a rhodamine derivative induced by Al^{3+} binding with such multiple logic gate operations accompanied by its application to cell-biology.

Introduction:

The design and synthesis of new sensitive and selective chemosensors have received a significant attention of chemists, biologists & environmental scientists which are widely used to spy on neutral and ionic species.[1] As is well-known, aluminum is the third most abundant element in the earth's crust and extensively used in modern society in water treatment, in food additives, in medicines, in the production of light alloys etc.[2] The leaching of aluminum from soil by acid rain increases the free Al^{3+} in the environment and surface water, which is deadly for the growth of plants.[3] Recent advances have shed light on the biological roles of aluminum, particularly of its functions related to neurotoxicity on human's health. Disorders of aluminum homeostasis are implicated in a number of diseases, such as Parkinson's disease (PD), Alzheimer's disease (AD), and dialysis encephalopathy.[4]

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Rickets, gastrointestinal problems, anemia, headaches, decreased liver and kidney function may also be caused by aluminum toxicity.[5] According to WHO report, the average daily human intake of aluminum is ~ 3–10 mg kg^{-1} and the tolerable weekly dietary intake is 7 mg kg^{-1} body weight.[6] However, sensitive bioimaging of Al^{3+} in the cell is a prerequisite to understand the underlying mechanism about how it causes aluminum-induced human diseases.[4] Hence, looking on the potential impact of Al^{3+} ions on human health, the detection of Al^{3+} has attracted increasing interest in the areas of chemical and biological sciences, particularly, to monitor the concentration levels in the biosphere and to minimize direct affects on human health.

A number of techniques available for Al^{3+} estimation include chromatography[7], accelerator mass spectroscopy (AMS)[8], graphite furnace atomic absorption spectrometry (GFAAS)[9] , neutron activation analysis (NAA)[10] inductively coupled plasma-atomic emission spectrometry (ICP-AES)[11], inductively coupled plasma-mass spectrometry (ICP-MS)[12], laser ablation microprobe mass analysis (LAMMA)[13], electrothermal atomic absorption spectrometry (ETAAS)[14] etc but most of them require sophisticated instruments and time consuming for sample preparation protocols, and in some cases cost-effective too. On the contrary, fluorescence chemosensors are the best choices, as they qualify with high sensitivity, fast response, and inexpensive

installations,[15] Hence, recently the design and synthesis of Al^{3+} selective fluorescent probes has received intense attention of the chemists.[16,17,28]

Compared to other transition metal ions, Al^{3+} fluorescent chemosensors are limited, may be, due to its poor coordination ability and lack of spectroscopic characteristics.[18] It has been found that Al^{3+} being a hard-acid prefers a hard coordination sphere of N and O donor atoms.[17,28] Not only that most of the reported Al^{3+}-sensors involve complicated synthetic routes with harsh reaction conditions and expensive chemicals. Therefore, it is important to develop an easily synthesizable selective and sensitive chromo/fluorogenic dual signaling probe for Al^{3+} with bio-imaging possibilities.

The rhodamine framework is an useful platform to construct OFF-ON fluorescent chemosensors due to its particular structural property.[19] Moreover, the rhodamine fluorophore provides good photo-stability, high extinction coefficient and a longer emission wavelength (> 550 nm), which are often preferred to avoid background fluorescence (below 500 nm).[19,20] In majority of the cases, it provides in-field detection via distinct optical colour change as well as the edge in imaging studies via OFF-ON fluorescent property. Because of all the above mentioned advantages rhodamine based fluorosensors have become an attractive choice for the design of receptors for various metal ions.[19]

Since the first pioneer work on 'AND logic gate' based on optical signals by de Silva et al,[21] the molecular logic gates become one of the interesting and encouraging research focus of the chemists for further miniaturization in information technology. Various molecular logic devices, such as logic gates (AND, OR, XOR, INHIBIT, NAND, half-adder, half-subtractor)[22], molecular keypad locks[23], information storage devices [24] etc have been widely explored in recent years. Such logic instruments are believed to transfer the molecular-level information through the observable optical signals[25], the integration of which into arithmetic systems has brought the chemists closer to the realization of a molecular scale calculator (moleculator).[26] Such devices operate in wireless modes and have the potential for computation on a molecular level, which cannot be addressed by silicon based devices. Hence, they have possible implications in the development of electronic and photonic devices in future. Moreover, recently Keypad locks, an important electronic logic device which can be opened only by the proper combination and sequence of chemical inputs, have attracted attention of the chemists. It allows accessing a secret message using a password restriction to a limited number of persons having the exact password to open the lock. Such molecular devices, capable of authorizing password entries, are of significance for information technology.[27] So, the idea to use molecular systems for information processing has attracted a great deal of interest in the recent year.

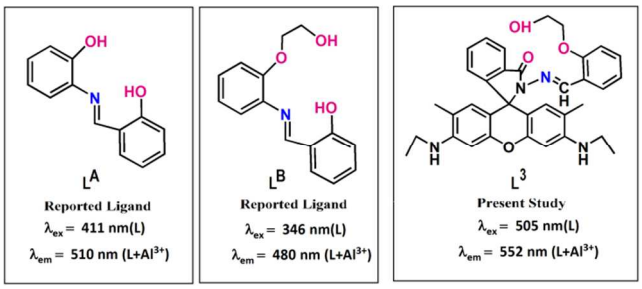


Fig.1 Different Al^{3+} sensors with N, O donor receptor sites.

Therefore, there is a demand to develop new synthetic strategies, usually employing a structurally well-developed receptor unit in the fluoro-ionophore that provides a reversible colorimetric as well as fluorescent turn-on responses essential for investigating intracellular Al^{3+} ion with no or negligible cytotoxicity. In particular, such fluorescent chemosensor can be used not only for detection of Al^{3+} in aqueous buffer-methanol solution and in biological cells, but also to integrate metal ion as chemical-driven molecular ''intelligence" machines in the future molecular computing. Towards this end, we present, here, a new type of simple and easily synthesizable salicylaldimine-based receptor with potential hard N,O donor atoms equipped with rhodamine fluorophore for sensitive and rapid recognition of Al^{3+} ion (**Fig. 1**). Very recent reports by J. Kim and A-T Wu groups[28] on Al^{3+} sensitive probes bearing N,O donor atoms have inspired us to design a probe with salicylaldimine-based N, O, donor receptor and rhodamine as fluorophore for preferential recognition of Al^{3+}. Our modification of incorporation of rhodamine unit shifts both the excitation and emission wavelengths towards lower energy side (≥ 500 nm) thereby improving its biocompatibility for the *in vitro*/*in vivo* monitoring of Al^{3+} compared to the existing Al^{3+} probes. Besides, sequence dependent logic gate operations namely 'OR', 'Key-pad lock' and 'INHIBIT' open up a new channel for potential applications in the development of new generation molecular devices.

Results and discussion

Sensor **L^3** was easily synthesized by simple Schiff base condensation between Rhodamine-6G hydrazide (**L^1**) and 2-(2-hydroxyethoxy**)** benzaldehyde (**L^2**) in MeOH with satisfactory yield (77 %) (**Fig. 2**) and thoroughly characterized by ESI-MS^+ (m/z), ^1H, ^{13}C-NMR and CHN analyses. The characteristic ^{13}C-NMR peak at 65.42 ppm corresponding to C4 in **L^3** suggests that it exists in solution predominantly as colorless and fluorescence inactive spirolactam form.[30] A visual change in solution color from colorless to orange-yellow (**Fig. 3 (a)**) clearly demonstrates the formation of the ring-opened amide form of **L^3** upon binding with Al^{3+}.[19]

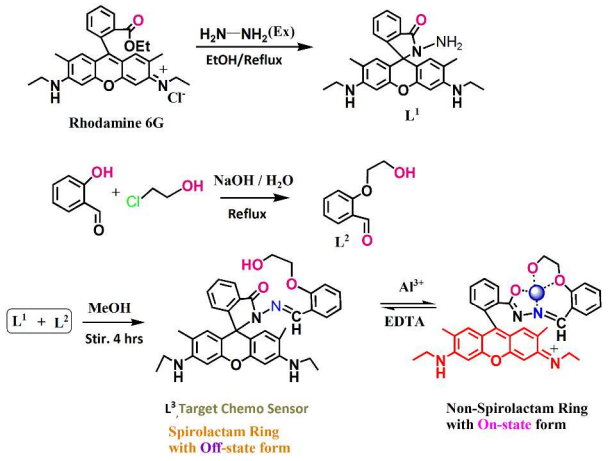


Fig. 2. Synthetic routes of chemosensor (L^3) and its binding mode towards Al^{3+}

The spectrophotometric titration for the interaction of **L^3** with Al^{3+} at 25°C in aqueous buffer-methanol solution (3:7 v/v, HEPES buffer, pH 7.0) shows a gradual development of a new absorption band centered at ~530 on gradual addition of Al^{3+} in the range 0-60.0 μM [Fig. 4 (a)] and it

gets saturated upon addition of ~1.30 equivalent of Al^{3} keeping the concentration of **L^{3}** fixed at 20.0 μM. So, the chemosensor L^{3} can indeed serve as a highly sensitive 'naked eye' indicator for Al^{3++}, but cannot be used in presence of Cu^{2+} (vide infra).

The fluorescence property of **L^{3}** was also investigated in aqueous buffer-methanol solution (3:7 v/v, HEPES buffer, pH 7.0) at 25^{0} c (**Fig. 5A**) in presence of Al^{3+}. On gradual addition of Al^{3+} (0–35 μM) to the non-fluorescent solution of **L^{3}** (7 μM), there is a gradual increase in fluorescence intensity at ~552 nm and becomes saturated on adding ~1.4 equivalent of Al^{3+} resulting ~180 fold fluorescence enhancement on excitation at 505 nm and this also clearly suggests the opening of spirolactam ring of **L^{3}** on binding to Al^{3+} ion. Selectivity is one of the most important features for a probe responding to a metal ion target.

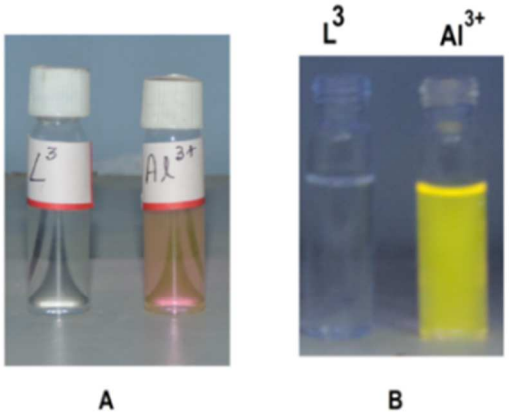


Fig. 3. Photograph of solution of L^{3} and the L^{3}–Al^{3+} complex. (A) for naked eye image; (B) for UV-exposed image by 366 nm lamp.

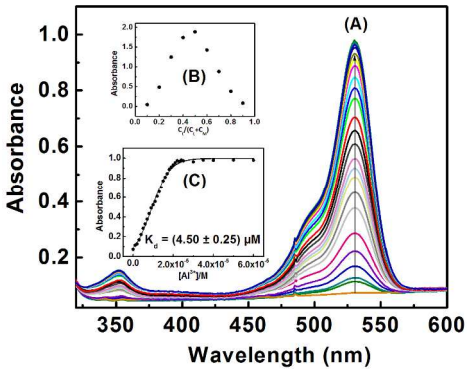


Fig. 4. (a) Change in absorption spectra of **L^{3}** (20 μM) upon addition of Al^{3+} in HEPES buffer at pH 7.0 in H_2O–MeOH = 3:7 (v/v) at 25 °C, [Al^{3+}] = 0–60 μM; (b) 1:1 binding stoichiometry shown by JOB's plot (c) non-linear plot of absorbance (at 530 nm) vs. [Al^{3+}] for the corresponding Uv-Visible titration.

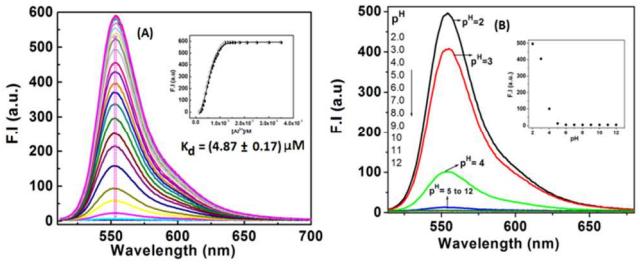


Fig. 5 (A) Change in fluorescence intensity of **L^{3}** (7μm) upon addition of Al^{3+} in HEPES buffer at pH 7.0 in H_2O:MeOH (3:7, v/v) at 25 °C with [Al^{3+}] = 0 - 35 μm (λ_{ex} = 505 nm); inset: non- linear plot of fluorescence intensity (at 552 nm) vs. [Al^{3+}] for this titration. (B) pH–stability study of the sensor **L^{3}** (6 μm); inset: fluorescence intensity vs. pH plot at 554 nm.

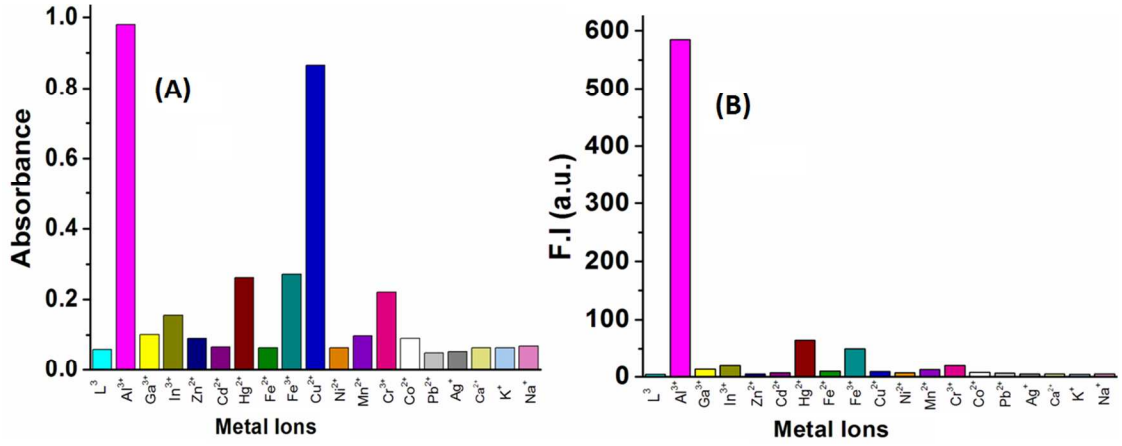


Fig. 6 Bar chart illustrating (a) absorption response at 530 nm with [**L^{3}**] = 20 μM and (b) fluorescence response at 552 nm (λ_{ex} = 505 nm) with [**L^{3}**] = 7 μM in the presence of 5 equivalent of different camions (M^{n+} = Al^{3+}, Ga^{3+}, In^{3+}, Zn^{2+}, Cd^{2+}, Hg^{2+}, Fe^{2+}, Fe^{3+}, Cu^{2+}, Ni^{2+}, Mn^{2+}, Cr^{3+},Co^{2+}, Pb^{2+}, Ag^{+},Ca^{2+}, K^{+}, Na^{+}) in HEPES buffer at pH 7.0 in H_2O: MeOH (3:7, v/v; at 25^{0}C).

Al^{3+} detection was not perturbed by biologically abundant Na^+, K^+, Ca^{2+} etc metal ions. Except Cu^{2+}, Fe^{3+}, Cr^{3+} and Hg^{2+} other interfering metal ions like Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+} Cd^{2+}, Pb^{2+}, Ag^+, In^{3+} and Ga^{3+} become almost innocent towards UV-Vis absorption change. [**Fig. 6(A) and Fig S1a** in ESI†]. Moreover, no significant change in emission spectra was observed even after addition of excess (5 equivalents) of the above mentioned interfering metal ions including Cu^{2+} and Cr^{3+} as shown in **Fig. 6 (b)** and **Fig. S1b** in ESI†. In case of Cu^{2+} that strongly responds calorimetrically, the fluorescence of the opened ring form of L^3 was fully quenched by Cu^{2+} due to its well known paramagnetic (d^9) effect making the system silent toward emission.[31a,b] The reduced fluorescence specificity of the sensor towards Al^{3+} was due to the slight responses towards Fe^{3+} and Hg^{2+}. The fluorescence enhancement (FE) are: 180, 13, and 19 folds for $Al^{3+,}$ Fe^{3+} and Hg^{2+} respectively, which clearly demonstrates the preference of the probe towards Al^{3+} over the others. The paramagnetic quenching effects of Fe^{3+} and Cr^{3+} may be responsible for this reduced FE[31c-f] of the opened ring form of L^3 while in case of Hg^{2+} ion, this reduced FE is probably due to heavy metal ion effects along with the lack of soft centre in the ligand framework.[31g,h]

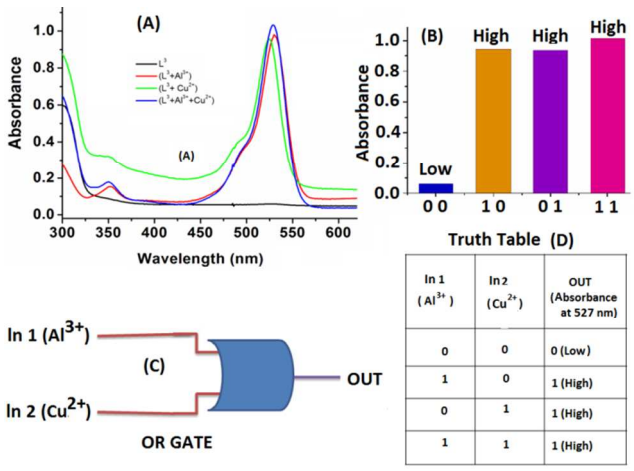
The above discussions indicate that the oxophilic nature and high charge density of Al^{3+} favored strong complexation with the rhodamine based non-fluorescent probe L^3 leading to the opening of the spirolactam ring and thereby resulting an intense fluorescence enhancement. The dissociation constant (K_d) was determined by fitting the absorption data as a function of metal ion concentration to a suitable computer-fit non-linear program and evaluated to be K_d = (4.50 ± 0.25) μM for Al^{3+}(**Fig. 4**c, inset). Similarly the non-linear fitting of fluorescence-titration data gives K_d = (4.87 ± 0.17) μM Al^{3+} ion. (Fig. **5**A, inset). There is an excellent agreement between the K_d values for L^3-Al^{3+} system obtained from absorption and fluorescence titrations and clearly manifests the self-consistency of our results. The composition of the complex found to be 1:1 determined by Job's method (**Fig. 4b**) and also further supported by mass spectrometric analysis with m/z = 627.27 $[Al(C_{35}H_{35}N_4O_4)(H_2O)Li]^+$ (see **Fig. S2 in** ESI†). The fluorescence responses of the probe towards Hg^{2+} and Fe^{3+} were investigated fluorescence titration and corresponding dissociation constants (K_d) were determined to be 0.31 μM and 0.26 μM, respectively (see Fig. S3 in ESI†). In case of Cr^{3+} the fluorescence enhancement is too small (~ 3 fold) to be used for the determination of K_d.

Now, for practical application, the appropriate *p*H conditions for successful operation of the sensor were evaluated. No obvious fluorescence emission of $\mathbf{L^3}$ was observed between pH 5 and 12, suggesting that the non-spirolactam form of $\mathbf{L^3}$ was stable over this wide range of pH and can work well at physiological pH conditions [Fig. 5(B)]. A short response time and reversible response are also necessary for a fluorescent sensor to monitor Al^{3+} ions in practical applications. The time dependence of response of $\mathbf{L^3}$ towards Al^{3+} ion was investigated by Uv-Vis spectrophotometry and the results revealed that the recognition event could complete almost immediately upon mixing (≤1.0 min) (Fig. S4).

Reversibility is one of the important parameter to satisfy the demand of a novel chemosensor particularly for improving practical real time applications of a probe. So, the reversible binding of $\mathbf{L^3}$ with Al^{3+} was checked using EDTA as a strong chelating ligand in identical reaction conditions (**Fig. S5 in ESI†**). The addition of EDTA to the reaction mixture of L^3 and Al^{3+} causes demetalation of Al^{3+} from the corresponding complex and regeneration of the spirolactam ring resulting the bleaching of absorption band at 530 nm and the emission band at 552 nm. The quantum yield determination of the $\mathbf{L^3}$–Al^{3+} complex gives Φ= 0.721 (with rhodamine 6G used as a standard), whereas the free ligand is non- or very weakly fluorescent with a very negligible absorption band at 530 nm. The limit of detection (LOD) of Al^{3+} was determined by the 3σ method [33] and found to be as low as ~73.0 pM (**Fig S6**).All these findings indicate that the chemosensor $\mathbf{L^3}$ behaves well for Al^{3+} recognition.

The proposed mechanistic pathway of the formation of $\mathbf{L^3}$- Al^{3+} complex via opening of spirolactam ring was established through ^{1}H, ^{13}C-NMR and IR studies. IR studies revealed that the characteristic stretching frequency for the 'C=O' in amide group of the rhodamine moiety at 1699 cm^{-1} was shifted to lower wave number of 1645 cm^{-1} in the presence of 1.4 equiv of Al^{3+} (**Fig. S7 in ESI†**). The larger shift towards lower wave number signifies a higher polarization of the C=O bond upon efficient binding to the Al^{3+} ion indicating the cleavage of the above mentioned bond. Also in ^{1}H NMR, the ring protons (f and g; see the labeling in **Fig. S8 in ESI†**) of the Rhodamine part moved to the downfield direction ($\Delta\delta_f$ = 0.06 **ppm**, $\Delta\delta_g$ = 0.03 **ppm**) in the presence of 1.4 equiv. of Al^{3+} ions. The 'p' proton showed a down field shift ($\Delta\delta_p$ = 0.28 **ppm**) mainly due to the rotation of the molecule in the presence of Al^{3+}.[16a] Also in ^{13}C-NMR, the disappearance of the signal at 65.42 ppm for the tertiary carbon (sp^3 hybridized) of the spirolactam ring of L^3 (labeled as C4, see **Fig. S9**) upon addition of 1.4 equiv. of Al^{3+} convincingly supports the opening of spirolactam ring and coordination through O atom of -CONH group of the rhodamine moiety with Al^{3+} ion.

When we consider the ligands L^A, L^B and L^3 it is found that the receptors in all these ligands are almost same and all of them recognize Al^{3+} in preference to other metal ions. The ligand L^A senses Al^{3+} (λ_{ex} = 411 nm, λ_{em} = 510 nm) with K_D = 32 μM that also provides intracellular application, but with no selectivity, as it recognizes Ga^{3+} too, which belongs to the same group with Al^{3+}, with significant FE. While the ligand L^B acts as turn on sensor for Al^{3+} (K_D = 1.9 μM) along with λ_{em} = 480 nm when it was excited at 346 nm but with no cell studies. The present probe (L^3) is superior to others based on the fact that it can be excited at 505 nm without background interference. Not only that the K_d value (4.5 μM) lies well within the limit of mM-nM range for the Al^{3+} probes reported so far. Besides, the probe L^3 being biocompatible with respect to the both the excitation and emission wavelengths towards lower energy side (≥ 500 nm) provide intracellular application without cytotoxicity effects which is already discuss later.



In 1 (Al^{3+})	In 2 (Cu^{2+})	OUT (Absorbance at 527 nm)
0	0	0 (Low)
1	0	1 (High)
0	1	1 (High)
1	1	1 (High)

Fig. 9 (A) Absorbance Output signals (at 527 nm) of the logic gate in presence of different inputs with corresponding bar diagram(B); (C) general representation of the symbol of an OR gate (D) corresponding truth table of the logic gate.

We observed that the paramagnetic Cu^{2+} showed significant calorimetric response towards L^3 with the development of a new absorption band centered at ~526 on gradual addition of Cu^{+}; although it was emission silent due to strong paramagnetic quenching by Cu^{2+} ion. Its dissociation constant [K_d =(5.50 ± 0.30) μM] obtained from UV-Vis titration was found to be slightly higher than that of Al^{3+}. Binding stoichiometry of the L^3-Cu^{2+} complex determined by JOB's plot was found to be 1:1 (Fig. S10) which is further supported by the mass spectrometric analysis (m/z =638.18 [Cu $(C_{35}H_{35}N_4O_4)$] (Fig. S11). As the K_d values of L^3-Al^{3+} and L^3-Cu^{2+} are comparable, Al^{3+} and Cu^{2+} would compete against each other to coordinate with L^3 when they coexist in a system.

Based on the above observations, we investigated the different absorbance as well as fluorescence state ''ON and OFF'' of L^3 with changing the addition sequence for finding out some interesting chemistry related to mimicking advanced logic operations. The absorbance as well as fluorescence properties (output i.e. 1 for 'YES'/'ON-state' and 0 for 'NO'/'OFF-state') of L^3 caused by chemical inputs and the information thereby gathered can be interpreted within the framework of various molecular logic. Here, the optical output signals (based on O.D.) of L^3 in response to metal ions (Al^{3+} and Cu^{2+}) binding can be used to design OR gate.

Such '**OR**' gate is one of the basic logic gates that implement logical disjunction, which results in a high output if one or both inputs to the gate are high. The absorbance of L^3 (20 μM) at 527 nm can be enhanced giving rise to a output signal as 1 (ON-state) by the effect of Al^{3+} or Cu^{2+} or both, enabling the **OR** logic function when 1.5 equiv. of Al^{3+} and 5 equiv. of Cu^{2+} are taken as inputs (**Fig. 9)**

On the other hand, the fluorescence output of L^3 at 552 nm on proper sequencing addition of Al^{3+} and Cu^{2+} as inputs was utilized to fabricate another important molecular logic gate function; namely' **Keypad lock**' which allow to create a secret password. In the absence of any chemical input, the receptor L^3 shows no prominent emission band at 552 nm, i.e. output 0 (OFF-state) Now, for the first input sequence, Al^{3+} and Cu^{2+} were used as inputs A and B, respectively. Operation by Al^{3+} as (In A) to receptor L^3, gives the output "1" (ON-state) and with the sole of sequential addition of another input Cu^{2+} (in B), the output still remained '1' (ON-state) i.e. for both cases fluorescence intensity cross the threshold limit (120; indicated by dotted line). But, On reversal of the inputs sequence, i.e. for Cu^{2+} as the first input followed by Al^{3+} as the second one, it caused the fluorescence intensity far below its threshold limit (F.I. = 120) leading to 'Off' state as output '0'(**Fig. 10**).

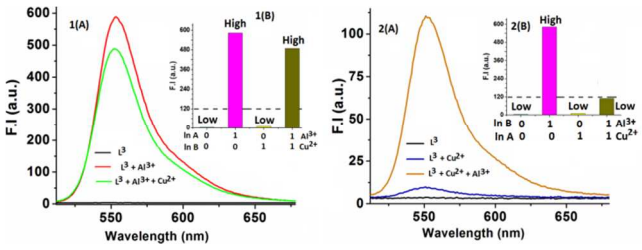


Fig. 10. (A) Emission output of L^3 following excitation at 505 nm with different input sequences: (1A) Al^{3+} as first input A followed by Cu^{2+} as second input B; (2A) Cu^{2+} as first input A followed by Al^{3+} as second input B. The corresponding emission output bar diagram at 552 nm is shown in an inset :(1B) In A (Al^{3+} first) and In B (Cu^{2+} second); 2B) In B (Cu^{2+} first) and In A (Al^{3+} second).The dotted line represents the threshold limit (F.I=120 nm) for the concern logic operation.

On the basis of this observation (**Fig. 10**) our system can be used as a password entry for a keypad lock; inputs Al^{3+} and Cu^{2+} were designated as 'S' and 'K' respectively.

Out of two possibilities of inputs: (a) first addition of 'S' followed by 'K' where the receptor (L^3) showed emission 'ON' at 552 nm and this "switch ON" is represented by 'Y' creating a secret code '**SKY**'; (b) While on the other hand, by reversing the order of inputs i.e. first input 'K' followed by 'S', the fluorescence at 552 nm remained "switch OFF" and is represented by 'X', which is a wrong entry (KSX), and failed to open the **keypad lock**. Thus, the sequence dependence of inputs is mandatory for the construction of the molecular **keypad lock** i.e. '**SKY**' only (**Fig 11**).

Therefore, only the authorized user who knows the exact password ''**SKY**'' can open the lock, which is a new approach for protecting information at the molecular scale. Due to the fact that the use of numerical digits (0–9) and letters (A–Z) as 'PIN' numbers in a two-digit password allows a total of more than 700 different combinations[23b], this adds to the complexity of cracking the keypad lock, and improved remarkably the security of the molecular devices.

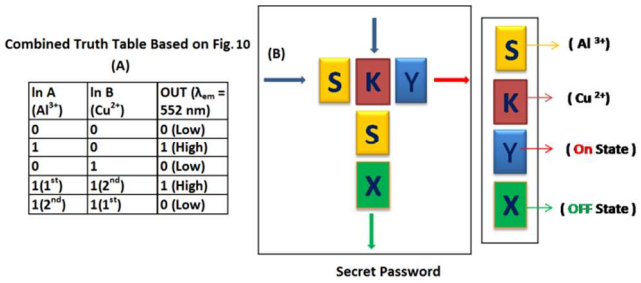


Fig. 11. (A) Truth table for Keypad logic gate operation.**(B)** Fluorescent **keypad** to access a secret password at 552 nm with different input sequences.

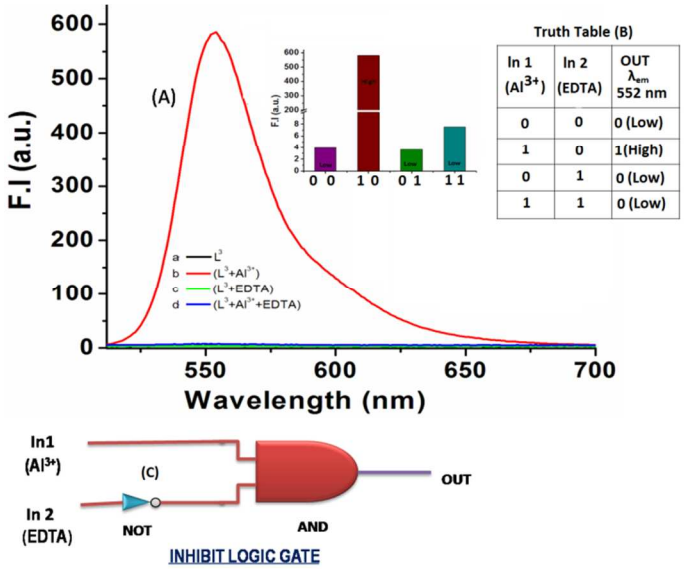


Fig. 12. (A) Output signals (at 552 nm) of the logic gate in presence of different inputs; (B) corresponding truth table of the logic gate; (C) general representation of the symbol of an INHIBIT gate.

Moreover, we find out another logic gate operation; namely INHIBIT logic gate which involves a particular combination of LOGIC functions AND and NOT. For our system we correlate it by taking two input signals namely Input 1 (Al^{3+}) and input 2 (EDTA) along with fluorescence signal of the probe **L^3** at 552 nm as output. For input, the presence and absence of Al^{3+} and EDTA is assigned as 1(on-state) and 0 (off-state) respectively. For output, we assign the enhanced fluorescence of **L^3** as 1 (on-state) and the quenched fluorescence as 0 (off-state) (**Fig. 6B**, Truth table). In the absence of both inputs (Al^{3+} or EDTA) **L^3** remains in off-state form. Now, input 1 leads to significant fluorescence enhancement with interaction with free receptor in its occupied state leading to on-state. But on subsequent addition of input 2 interacting with the corresponding receptor L^3 leads to off-state, thereby implementing the necessary NOT gate. The probe acts in parallel on the fluorescence output signals, which implements the required AND function. Now the combination of both inputs lead to the fluorescence quenching as output 0, in accordance with the truth table in **Fig. 12B**. Therefore, monitoring the fluorescence at 552 nm, upon addition of Al^{3+} and EDTA and their combined mixture lead to satisfy an INHIBIT logic gate function (**Fig. 12**).

The intracellular Al^{3+} imaging behaviours of **L^3** were studied on A375 cells with the aid of fluorescence microscopy. After incubation with **L^3**, the cells displayed no intracellular fluorescence (**Fig. 13B**). However, exhibited intensive fluorescence when the cells pre-incubated with **L^3** were added with Al^{3+} ion (10μM - 50μM) and the fluorescence intensity is also dependent on added [Al^{3+}] (**Fig. 13 C-F**). Thus, this fluorescence response of **L^3** towards Al^{3+} ion can have specific application in monitoring Al^{3+} in different biological conditions in the concentrations range ≥ 73 pM present in the cells. The minimum concentration of Al^{3+} that can be detected by the sensor in cells is about 0.50 μM, as evidenced from the exposure of 0.25, 0.50 and 0.75 μM of Al^{3+} with cells pre-incubated with 10 μM of the probe (Fig. S12).

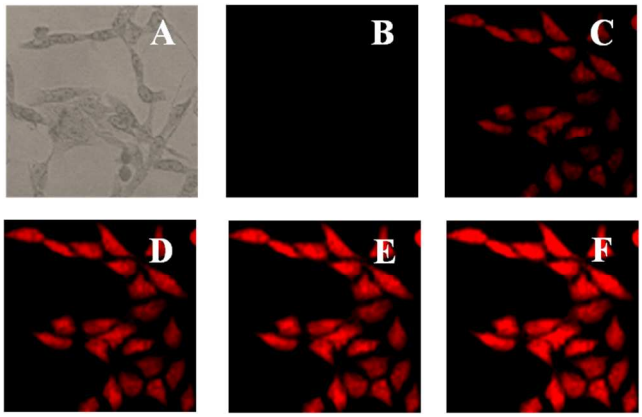


Fig. 13. Cell imaging experiments: (A) Phase contrast image, (B) A375 cells after being incubated with 10 μM of L^3 only for 30 min at 37 °C and washed with PBS buffer. Cells were then exposed sequentially with increased extracellular Al^{3+} ion at a concentration and imaged: (C) 10 μM, (D), 20 μM (E), 30 μM (F) and 50 μM (F), respectively. For all imaging, the samples were excited at 505 nm.

Not only that, MTT assay results of free **L^3** (10 μM) up to 12 hr and the MTT assay results of the **L^3** (10 μM, a) with different concentrations of Al^{3+} ions (10 (b) , 20(c) , 30(d) and 50(e) μM) proved its relatively low cytoxicity (**Fig. 14 A, B**). Hence this finding indicates that it can be readily used as a chemosensor to detect the presence of Al^{3+} ions at the indicated doses and time of incubation without much worry about its cytotoxicity

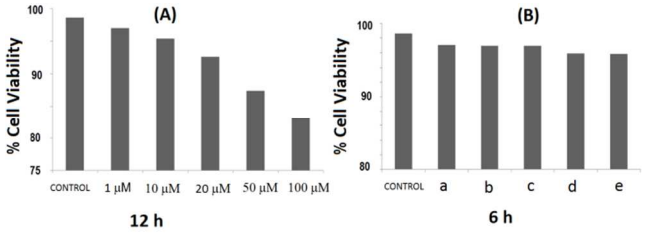


Fig. 14 (A) depicts % cell viability of A375 cells treated with different concentrations (1 μM-100 μM) of L^3 for 12 hours determined by MTT assay. Results are expressed as mean of three independent experiments. **(B)** represents % cell viability of A375 cells treated with fixed concentration [10 μM (a)] of L^3 along with different concentrations [10 μM (b), 20 μM (c), 30 μM (d), 50 μM (e)] of Al^{3+} salts for up to 6 hrs determined by MTT assay. Results are expressed as mean of three independent experiments.

Conclusions

In summary, we have designed and synthesized a simple new rhodamine-based chromo-/fluorogenic dual signaling probe for preferential recognition of Al^{3+} which has been characterized by ^1H, ^{13}C-NMR, ESI-MS (m/z) studies. The binding stoichiometry of the sensor with Al^{3+} was established by the combined UV-vis, fluorescence and MS(m/z) method. All biologically relevant metal ions and toxic heavy metals such as Cd^{2+} and Pb^{2+} did not interfere with the Al^{3+} ion detection. The detection limit of Al^{3+} calculated by 3σ method gives a value of 24.8 pM. Owing to its bio-compatible nature with respect to its good solubility in aqueous buffer-methanol media ($MeOH/H_2O$) along with its cell permeability without no or negligible cytotoxicity provide good opportunity towards *in-vitro*/ *in-vivo* cell imaging studies. In addition, the color and fluorescence responses of the probe upon metal binding were employed for developing logic gate operations like for '**OR**' and '**Keypad Lock**' logic functions by the two chemical inputs (Al^{3+} and Cu^{2+}) in the proper sequencing way. Such fluorescent lock may be used for security devices, which would allow access using specific ionic keys as a password. Not only that, the 'OFF–ON–OFF' fluorescence sensing behavior observed in the presence of Al^{3+} and EDTA strengthens potential applications of the L^3–Al^{3+} system as a device with '**INHIBIT** logic gate functions on controlling condition. Therefore, all the advantages related to such multiple logic gate functions may be helpful for the development of new-generation digital devices. Such interesting works are in progress in our laboratory.

Experiment section

Materials and Methods

Physical measurements. Steady-state fluorescence studies were carried out on a Shimadzu spectropfluorimeter (Model RF-5301PC). UV-vis absorption spectra were recorded on a diode array spectrophotometer (Model: Agilent 8453). NMR spectra were recorded in Bruker instrument (300 MHz). The ESI-MS^+ spectra were recorded on a QTOF Micro YA263 mass spectrometer. Cell images were taken using Leica filter N2.1 (Excitation filter-BP 515-560, Dichromatic mirror- 580, Suppression filter LP-590.

Materials

All solvents used for the synthetic purpose were of reagent grade (Merck) unless otherwise mentioned. For spectroscopic (UV-Vis. and fluorescence) studies HPLC grade MeOH and double distilled water were used. Rhodamine 6G hydrochloride, 2-chloroethanol and Metal salts such as perchlorate of Na^+, K^+, Ca^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Pb^{2+}, Cd^{2+}, Hg^{2+}, and $Cu(NO_3)_2.2H_2O$, $Al(NO_3)_2,9H_2O$ were purchased from Sigma-Aldrich and used as received. All other compounds were purchased from commercial sources and used as received.

Preparation of Rhodamine-6G hydrazide (L^1):

Rhodamine-6G hydrazide was prepared according to a literature method.[34]

Preparation of 2-(2-hydroxyethoxy)benzaldehyde (L^2):

It was prepared by following a literature procedure.[35] Salicylaldehyde (1.22 g, 10 mmol) was added dropwise to a vigorously stirred solution of sodium hydroxide (0.4 g, 10 mmol) in water (10 ml) over a period of 15 min and then 2-chloroethanol (0.81 g, 10 mmol) was added dropwise. The resulting solution was heated at 98 °C for 16 h. The solution was cooled and maintained at about 10 °C while sodium hydroxide was added until the solution was alkaline to pH 10.The reaction mixture was extracted with dichloromethane (4 x 20 ml). The combined organic layers were dried over anhydrous $MgSO_4$ and then filtered. The filtrate was evaporated to dryness under reduced pressure giving rise to a yellow oil which was subjected to chromatography using 5:1 petroleum ether/ EtOAc to afford the corresponding desired product of 2-(2-hydroxyethoxy)benzaldehyde as a faint yellow oil (Yield 72%). ^{1}H-NMR (in $CDCl_3$) (δ, ppm): 10.48 (s, 1-H),7.84 (dd, 1-H), 7.56 (td, 1-H); 7.1 (m, 2-H); 4.03-4.23 (m, 4-H). (**Fig. S13a**).^{13}C-NMR (in $CDCl_3$) (δ,ppm): 190.33, 160.96, 136.99, 133.74, 124.98, 121.03, 112.94, 70.22, 61.13 (**Fig. S13b**) ES^+-MS (m/z): 189.07 ($L+Na^+$) (**Fig. S13c**)

Preparation of L^3.

To a 1 mmol (0.428 g) of L^1 in 30 ml MeOH, with one drop of acetic acid, 1.1 mmol of L^2 (0.183 g) in 10 ml MeOH was added dropwise for 30 minutes in hot condition. Then the entire reaction mixture was kept on stirring for around 4 h at room temperature. Off-white precipitate thereby formed was collected by filtration. The residue was washed thoroughly with cold ether/methanol (mixture) to isolate L^3 in pure form with 77% yield.

M.P. 172.6 ºC. Anal. Calcd for $C_{35}H_{36}N_4O_4$: C, 72.90; H, 6.29; N, 9.72. Found: C, 72.88; H,6.35; N 9.76. ^{1}H-NMR (in DMSO-d_6) (δ, ppm): 8.605 (s,1-H), 7.86(d, 1-H), 7.52-7.60 (m, 3-H); 7.27 (t, 1-H); 6.86-6.98 (m ,3-H), 6.29 (s, 1-H), 6.13 (s ,2-H), 3.87 (t, 2-H), 3.73 (t , 2-H), 3.13 (q, 4-H), 2.48 (s ,6-H), 1.19 (t, 6-H) (**Fig. S14a**).^{13}C-NMR (in DMSO-d_6) (δ, ppm): 143.22, 157.53, 152.52, 151.20, 148.24, 141.15, 134.24, 131.92, 129.05, 128.67, 125.09, 123.40, 121.16, 118.70, 113.47, 104.95, 96.31, 70.19, 65.42, 59.97, 39.65, 17.42, 14.61 (**Fig. S14b**). ES^+-MS (M/Z): 577.31 ($L+H^+$) (**Fig. S14c**). IR: ν˜ = 1698.93 (spirolactam amide keto), 1622.82 (–C=N), 1261.34 (aliphatic C–O) cm^{-1} (**Fig. S14d**).

Cell imaging experiment and cytotoxicity studies

Cell culture.

Cells belonging to A375 human melanoma cell line were procured from National Center for Cell Science, Pune, India, and used as materials throughout the study. Cells were cultured in DMEM (Gibco BRL) supplemented with 10% FBS (Gibco BRL), and 1% antibiotic mixture containing PSN (Gibco BRL) at 37 °C in a humidified incubator with 5% CO_2; cells were grown up to 80-90% confluence, harvested with 0.025% trypsin (Gibco BRL) and 0.52 mM EDTA (Gibco BRL) in phosphate-buffered saline (PBS), plated at the desired cell concentration and allowed to re-equilibrate for 24 h before any treatment.

Cell imaging study:

Cells were rinsed with PBS and incubated with DMEM containing L^3 making the final concentration up to 10 μM in DMEM [the stock solution (1 mM) was prepared by dissolving L^3 to the mixed solvent (DMSO: water = 1:9 (v/v)] for 30 min at 37ºC. After incubation, bright field and fluorescence images of A375 cells were taken by a fluorescence microscope (Model: LEICA DMLS) with an objective lens of 20X magnification. Fluorescence images of A375 cells incubated with 10 μM L^3 for 30 min followed by addition of different concentrations (10 μM-50 μM) of Al^{3+}ions were also taken.

Cell cytotoxicity assay:

To test the cytotoxicity of L^3, 3-(4, 5-dimethylthiazol-2-yl)-2,S-diphenyltetrazolium bromide (MTT) assay was performed as per the procedure described earlier[36]. After treatment with L^3at different doses of 1, 10, 20, 50 and 100 μM, respectively, for 12 h, 10 μl of MTT solution (10mg/ml PBS) was added to each well of a 96-well culture plate and again incubated continuously at 37ºC for a period of 3 h. All media were removed from wells and 100 μl of acidic isopropyl alcohol was added into each well. The intracellular formazan crystals (blue-violet) formed were solubilized with 0.04 N acidic isopropyl alcohol and absorbance of the solution was measured at 595 nm wavelength with a microplate reader (Model: THERMO MULTI SCAN EX). The cell viability was expressed as the optical density ratio of the treatment to control. Values were expressed as mean of three independent experiments. The cell cytotoxicity was calculated as % cell cytotoxicity = 100% - % cell viability.

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A simple rhodamine-based dual signalling reversible molecular switch for recognition of Al(III) with Promising applications for advanced logic operations – 'OR', 'Keypad Lock' & 'INHIBIT' logic function and cell-imaging studies†.

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Rhodamine based chemosensor for rapid detection of Al^{3+} with 'OR','Keypad Lock & 'INHIBIT' logic gate applications and bio-studies.