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Carbazole–thiosemicarbazone–Hg(II) ensemble-based colorimetric and fluorescence turn-on toward iodide in aqueous media and its application in live cell imaging†

Ajit Kumar Mahapatra,* Jagannath Roy, Prithidipa Sahoo,‡ Subhra Kanti Mukhopadhyay§ and Amarnath Chattopadhyay§

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A carbazole–thiosemicarbazone–Hg^{2+} ensemble-based fluorogenic probe for detection of iodide in aqueous media is reported. The first fluorescent sensor for iodide anions was constructed based on the displacement approach. An 'ensemble' is able to selectively sense iodide over other anions followed by the release of 9-(butane-1-yl)-9*H*-carbazole-3,6-dihydrazinecarbothioamide to give a remarkable change of fluorescence turn-on signal at pH 7.4 under aqueous media. The practical use of an 'ensemble' was demonstrated by its application to the detection of iodide in the living cells.

Iodide plays an important role in several biological activities such as neurological activity and thyroid function.[1] The sodium-iodide symporter (NIS) is an important plasma membrane glycoprotein that mediates active I^- transport in the thyroid gland, which is the first step in thyroid hormone biogenesis.[2] Thyroid hormones are responsible for the regulation of several metabolic processes such as growth and maturation of organ systems.[3] The estimation of iodide anions is performed frequently to examine thyroid disorders in clinics. Hence the iodide content of urine and breast milk is often required for nutritional, metabolic, and epidemiological studies of thyroid disorder.[4] WHO recommendations for daily iodine intake (as iodide) for adults is 150 µg day^{-1}. Thus, there is a great demand for the development of synthetic receptors capable of selectively recognizing iodide over other anions. Although iodide is such a biologically important anion, only a few papers have reported on the recognition of iodide.[5]

Among these reported methods, fluorescent probes possess innate advantages over probes of other types because of their high sensitivity, specificity, simplicity of implementation and fast response times, offering application methods not only for *in vitro* assays but also for *in vivo* imaging studies.[6] In particular, the fluorescent sensors have been mainly focused on cation targeting and on their corresponding bioimaging studies in living cells.[7] However, bioimaging studies of fluorescent chemosensors toward anions have been rarely reported, probably because of low solubility in aqueous media, low selectivity, and low sensitivity.[8] Also, the development of fluorescent sensors for anions in aqueous media is a challenging task owing to the strong hydration nature of anions and the competition of water for the hydrogen bonding sites, which weakens the interactions of the sensors with the target anions.[9] One way to tackle this hurdle is by employing the displacement method,[10] in which the sensor-ligand-metal ion "ensemble" is nonfluorescent due to metal ion-induced fluorescence quenching. However, the addition of anions may release the sensor ligand into the solution with revival of fluorescence.

So far, a few detection techniques have been developed for iodide anions, such as titration,[11] ICP-MS,[12] capillary electrophoresis,[13] iodide-selective electrodes,[14] spectrophotometry,[15] fluorimetry,[16] and chemiluminescence methods.[17] Despite the significant development in this domain, its ensemble version and bioimaging studies have not yet been explored for iodide detection. Due to the structural simplicity of iodide, finding a new detection method has recently been of keen interest in molecular recognition research. It is known that thiosemicarbazone is applied in mercury recognition[18] and detection may be achievable with a mercury(II) coordinated thiosemicarbazone moiety followed by demetalization to generate fluorescence. Therefore we incorporated these two moieties with carbazole, expecting it to form a carbazole–thiosemicarbazone–Hg^{2+} ensemble-based sensor to recognize iodide in aqueous solution. Taking advantage of this "off-on" fluorescence sensing system, we also herewith present intracellular iodide detection in cultured *Candida albicans* cells.

The target compound **3** was readily synthesized in two steps as shown in Scheme 1. Compound **3** was synthesized by

Department of Chemistry, Bengal Engineering and Science University, Shibpur, Howrah 711103, India. E-mail: mahapatra574@gmail.com; Fax: +91 33 26684564; Tel: +91 33 2668 4561

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‡ Present address: Chemistry and Chemical Biology Department, Rutgers University, 610 Taylor Road, Piscataway, NJ 08854, USA
§ Department of Microbiology, The University of Burdwan, Burdwan, West Bengal, India

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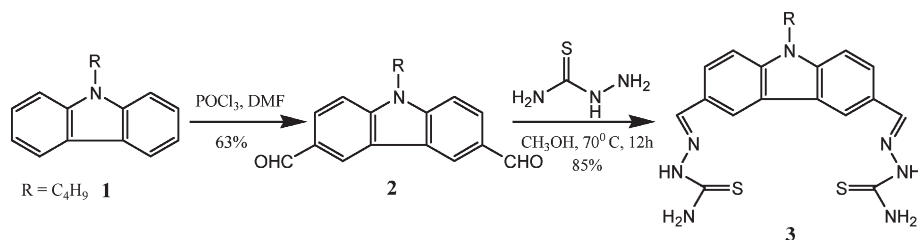

Scheme 1 Synthetic route to compound **3**.

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condensation of *N*-butylcarbazoledicarbaldehyde **2** and thiosemicarbazide in high yield, and its structure has been proved by various spectroscopic characterizations (see ESI†).

With compound **3** in hand, we examined its optical properties in the absence or presence of various heavy and transition metal species. The free compound **3** exhibited an absorption band at around 316 nm in pH 7.4 HEPES buffer (30 mM, pH 7.4, containing 1.0% DMSO). Upon gradual addition of $HgCl_2$ to a solution of **3** in HEPES buffer, induces a 94 nm red shift of the absorption from 316 to 410 nm and a perceived color change from colorless to yellow (Fig. 1). Two clear isosbestic points were observed at 270 and 384 nm, which is consistent with the presence of only two species, free ligand, and Hg^{2+}–ligand complex. No obvious responses could be observed upon the addition of Na^+, K^+, Cu^{2+}, Co^{2+}, Pb^{2+}, Al^{3+}, Mg^{2+}, Ni^{2+}, Cd^{2+}, Fe^{3+} and Zn^{2+}, respectively (Figure S1†). These results clearly suggested that the metal complexation of **3** show a great preference for the mercury ion over other cations. The binding constant was determined to be $(3.94 \pm 0.65) \times 10^4$ M^{-1} using non-linear regression analysis (Figure S2†).[19]

The corresponding fluorescence spectra have been measured for the receptor's solution (10.0 mM, pH 7.4, containing 1.0% DMSO) in the absence and presence of various metal ions. The free compound **3** is fluorescent with an emission peak at around 425 nm (Φ_{fl} = 0.71).[20] From the Fig. 2 and its inset, it can be seen that the intense emission peak at 425 nm is nearly quenched upon addition of 1 equivalent of Hg^{2+}, concomitant with a new weak band at around 362 nm and a distinct isoemissive point at 376 nm was observed, which may be ascribed to the ICT (internal charge transfer) effect.[21]

However, addition of heavy and transition metal ions caused fluorescence quenching to a different extent as shown in Fig. 2b. Compound **3** is selective for Hg^{2+} with fluorescence switching 'off' upon complexing the ion due to the MLCT-based[22] heavy metal ion effect. In good agreement with this finding, the Job plot also shows the formation of a 1 : 1 bonding mode between **3** and Hg^{2+} ions (Figure S3a†). Based on the 1 : 1 binding mode, the binding constant derived from the fluorescence titration data was found to be $(1.39 \pm 0.51) \times 10^5$ M^{-1} (Figure S3b†). The 1 : 1 binding model of Hg^{2+} and **3** can be further confirmed by mass spectra. The ESI mass spectrum of complex **3**–Hg(II) has a major peak with *m*/*z* of 624.9 $[\mathbf{3}\text{–Hg(II)}]^{+2}$, which corresponds to 1 : 1 complex (Figure S4†).

Based on the results of titration experiments and mass spectrum of **3**–Hg^{2+} complex, the carbazole–dithiosemicarbazone–Hg^{2+} ensemble-based sensor was prepared by mixing equal equivalents of compound **3** and mercuric chloride in the solution of HEPES buffer (50 mM, pH 7.4, containing 1.0% DMSO). We

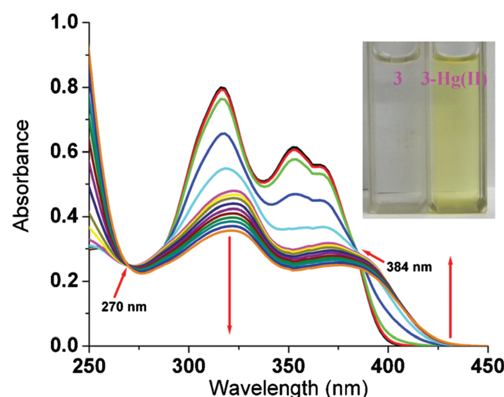

Fig. 1 UV-vis spectra of compound **3** (10 μM) with the increasing concentrations of Hg^{2+} ions (0–2 equiv.) in pH 7.4 HEPES buffer (25 mM, pH 7.4, containing 1.0% DMSO). Inset shows the change in color of compound **3** (10 μM) upon addition of Hg^{2+} ions (10 μM).

found that the **3**–Hg^{2+} complex is initially nonfluorescent (Φ_{fl} = 0.02), and the fact that Hg^{2+} can coordinate with iodide anions to form the stable species, HgI_2 (solubility product constant, $K_{SP} = 4.07 \times 10^{-29}$, reported value of $K_{SP} = 2.9 \times 10^{-29}$),[23] rendered us to speculate that the **3**–Hg^{2+} ensemble is promising as a turn-on fluorescent sensor for iodide anions. To test this idea, the absorption spectrum of the **3**–Hg^{2+} ensemble was recorded by gradual addition of iodide to a solution of **3**–Hg^{2+} in HEPES buffer. As shown in Figure S5† the absorption band at 375 nm and led to a blue shift of the absorption band from 375 to 330 nm, which is essentially identical with the maximal wavelength of the absorption peak of the free **3**, indicating that addition of iodide anions to the ensemble resulted in the release of the free **3**. Consistent with this observation, treatment with iodide caused a significant fluorescence turn-on response at 425 nm (Fig. 3), and up to a 25-fold fluorescence enhancement (Φ_{fl} = 0.71) was observed.

Furthermore, the fluorescent intensities at 425 nm have an excellent linear relationship with the concentrations of iodide anions from 0.5–8 μM (Figure S6†), and the detection limit was calculated to 250 nM, indicating that the ensemble is highly sensitive to iodide anions. However, it is well known that iodide has intrinsic fluorescence quenching nature due to the heavy atom effect[24] and low charge density with the excellent electron donor ability. Also notable is that the iodide-induced emission features are almost identical with that of **3** in the absence of any guest species, indicating the observed fluorescence response arises from the regeneration of receptor **3**. This can be rationalized since the softer I^- replaces the neutral ligands of **3** and captures Hg^{2+} to form more stable species HgI_2.

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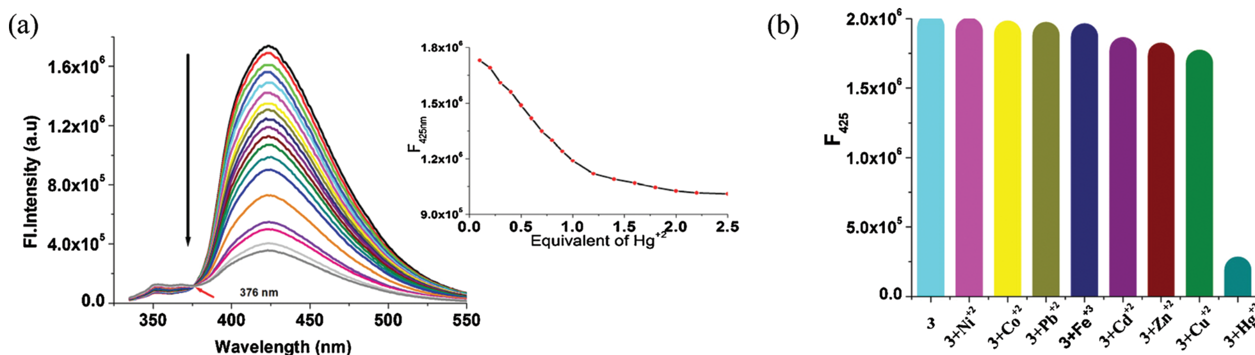

Fig. 2 (a) Fluorescence spectra of compound **3** (5 μM) with the increasing concentrations of Hg^{2+} ions (0–1.5 equiv.) in pH 7.0 HEPES buffer (25 mM, pH 7.4, containing 1.0% DMSO). The inset shows the fluorescence intensity changes at 425 nm of compound **3** (5 μM) with the amount of Hg^{2+} ions. (b) Bar diagram showing interaction of **3** with tested metal ions in the presence of HEPES buffer containing 1.0% DMSO at 425 nm.

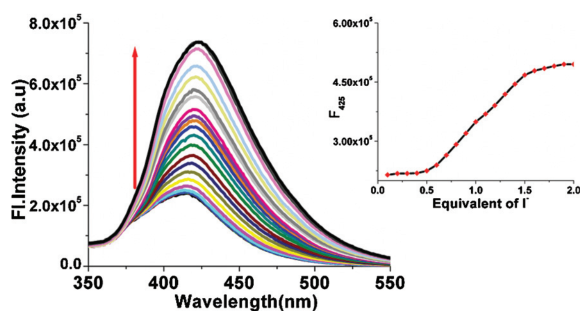

Fig. 3 Fluorescence spectra of the **3**–Hg^{2+} ensemble (5 μM) in pH 7.0 HEPES buffer(25 mM, pH 7.4, containing 1.0% DMSO) in the presence of iodide anions (0–2 equiv.). The inset shows the fluorescence intensity changes at 425 nm of the ensemble (5 μM) in the presence of increasing iodide concentrations (0–2 equiv).

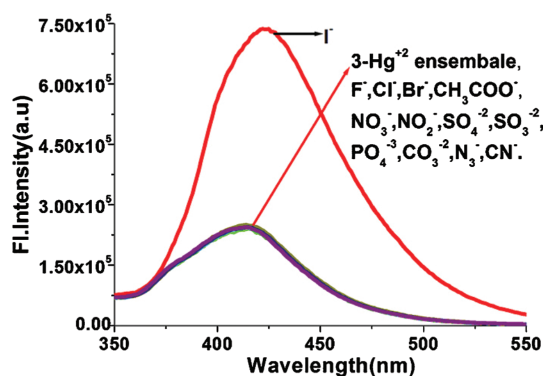

Fig. 4 Changes of the fluorescence spectra of the **3**–Hg^{2+} ensemble (5 μM) with anions (2000 equiv. of F^-, Cl^-, Br^-, NO_3^-,NO_2^-,N_3^-, SO_4^{2-}, SO_3^{2-}, CO_3^{2-}, PO_4^{3-}, and $CH_3COO^-$and 10 equiv. of CN^-; 2 equiv. for I^-) in pH 7.0 HEPES buffer (50 mM, pH 7.4, containing 1.0% DMSO).

To examine the selectivity, the **3**–Hg^{2+} in HEPES buffer ensemble (5 μM) was incubated with some representative anion species. As shown in Fig. 4, 2000 equivalents of F^-, Cl^-, Br^-, NO_3^-, NO_2^-, N_3^-, SO_4^{2-}, SO_3^{2-}, CO_3^{2-}, PO_4^{3-}, and CH_3COO^- and 10 equiv. of CN^- could not induce any marked fluorescence enhancement. By sharp contrast, 1 equiv. of I^- elicited a large fluorescence enhancement in the region of 425 nm. This sensory system demonstrates good selectivity toward iodide over many common anions. The sensor's fluorescence cannot be switched 'on' when presented with other halides (F^-, Cl^-, Br^-) or pseudohalides (SCN^-, N_3^-) due to the fact that the related species have soft or hard combination.

However, this detection method suffers from the interference of cyanide causing undesirable changes in fluorescence. Fortunately, these interferences could be reduced to some extent through an easy sample pre-treatment suggested by Bellack.[25] A possible chemical means of overcoming these interferences is to choose a metal that forms a very stable iodide complex. Provided this complex is more stable than the other metal ion complex, the formation of the latter will be suppressed and the interference of cyanide minimized. Thus, the spectroscopic studies suggest that the ensemble has a high selectivity for iodide over other test anions, which may be attributed to the low solubility product constant of HgI_2.

To further examine the I^--selective sensor, we also assessed the possible interference from commonly coexisting related cations and the potential competition of other relevant anions. The competitive experiments showed that most of the positively charged species, including ammonium ions (NH_4^+, n-Bu_4N^+), alkali metal ions (Na^+, K^+), alkaline earth metal ions (Mg^{2+}, Ca^{2+}) as well as others, such as Cu^{2+}, Al^{3+}, Cr^{3+}, Cd^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+}, do not produce substantial disturbance to the iodide sensing (Figure S7†). Similarly, the ensemble (5 μM) was treated with iodide anions (1 equiv.) in the presence of various test anions (2000 equiv. of F^-, Cl^-, Br^-, NO_3^-,NO_2^-,N_3^-, SO_4^{2-}, SO_3^{2-}, CO_3^{2-}, PO_4^{3-}, and $CH_3COO^-$and 10 equiv. of CN^-) in 50 mM, pH 7.4, containing 1.0% DMSO. As displayed in Figure S8,† all the relevant anions tested have virtually no influence on the fluorescence detection of iodide anions. Thus, the ensemble seems to be useful for selectively sensing iodide even involving these relevant anions. The fluorescence responses of the ensemble toward iodide anions were pH-dependent, and the maximal signal was observed in the pH range of 4.7–9 (Figure S9†). This indicates that the ensemble can be employed to sense iodide in a wide pH range.

NMR experiments were performed to explore the coordination and sensing mechanism between **3** and Hg^{2+}. The family of ^{1}HNMR spectra of receptor **3** obtained by the titration of Hg^{2+} are shown in Figure S10.† The continuous addition of Hg^{2+}

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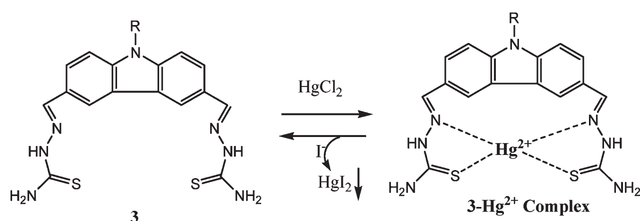
(from 0.5 to 1.5 equiv.) to the solution of receptor **3** caused a downfield shift in the signal corresponding to $^{-}$NH. No such significant changes were observed with the other proton signals, showing that Hg^{2+} is bound to the receptor through coordination of Hg^{2+} to the lone pair electrons on the sulfur atom and imine nitrogen.[26] It is well-known that Hg^{2+} ions (a soft acid) can preferentially interact with sulfur (a soft base) according to Pearson's hard and soft acids and bases theory.[27]

To gain insight into the sensing mechanism, we decided to study the sensing process by ^{1}HNMR spectra in presence of iodide ions. The product of **3** + Hg^{2+} + iodide anions was isolated by a silica gel column and was then subjected to ^{1}H NMR analysis. The ^{1}H NMR and mass spectra of the resulting product are essentially identical to that of free sensor **3**. Thus, the studies of NMR, mass spectrometry, absorption spectrometry, and fluorescence spectrometry indicate that the sensor likely functioned by the displacement mechanism (Scheme 2).



Scheme 2 Proposed displacement mechanism for sensing of iodide anions.

In order to further demonstrate that **3**–Hg(II), is able to detect the iodide ion in cells, the cells preloaded with **3**–Hg(II) complex were treated with various amounts of KI (Fig. 5). The cell permeability and sensitivity of compound **3** to Hg^{2+} was examined in living cells by using confocal microscopy.

Candida albicans cells (IMTECH No. 3018) incubated with **3** initially display a strong fluorescent image, the photographs indicate that the cells become green colored except their nucleus, so their nuclei are clearly detectable, but the fluorescence image immediately becomes faint in the presence of Hg^{2+} (Fig. 5b–d). The fluorescence intensities were again observed inside of the cells, after the KI ($c = 1.2 \times 10^{-6}$M) treatment. The bright field transmission images of these *C. albicans* cells in Fig. 5f is exactly the same as the fluorescence image in Fig. 5b, confirming that the imaged fluorescence is intracellular, instead of extracellular. The fluorescence intensities are apparently caused by the reaction of **3**–Hg(II) complex with I^{-} ion in the cells to give a regeneration of strongly fluorescent **3**. These results indicate that sensor **3** is cell membrane permeable and able to response to iodide in the living cells.

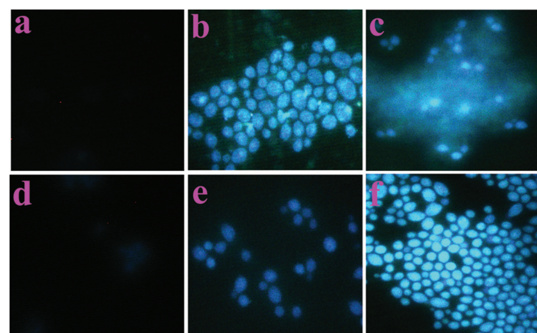


Fig. 5 (a) Fluorescence microscope images of *Candida albicans* cells only, (b) images of cells + **3**, (c) images of cells + **3** + Hg^{2+} (5 μM), (d) images of cells + **3**+ Hg^{2+} (25 μM), (e) images of cells + **3**–Hg^{2+} ensemble + KI, (f) same as (e) after 10 min.

Conclusion

In summary, we demonstrated that an "ensemble"-based **3**–Hg^{2+} in HEPES buffer can selectively probe the iodide ion in aqueous media with respect to a marked fluorescence enhancement over other anionic species under aqueous condition. The sensing ensemble is composed of a carbazole–semicarbazone, and mercury. The "off-on" fluorescence mechanism of **3**–Hg^{2+} with iodide ions is suggested as a decomplexation of Hg(II) to give strongly fluorescent **3** followed by light yellow precipitation of HgI_2. The selective iodide detection with **3**–Hg^{2+} ensemble for the biological application was also performed in *Candida albicans* cells to show the "off-on" fluorescence cellular image as well.

Experimental section

General procedures

Starting materials carbazole and thiosemicarbazide were purchased from Sigma–Aldrich Co. and used without further purification unless otherwise stated. UV-visible spectra were recorded using a JASCO V530 spectrometer. Fluorescence spectra were recorded using a Perkin-Elmer LS-55 fluorescence spectrometer. NMR and mass spectra were recorded on Varian instruments (300 and 400 MHz) and FAB MS mass spectrometer. For NMR spectra, $CDCl_3$, CD_3CN and d_6-DMSO was used as solvents, using TMS as an internal standard. TLC analyses were carried out on aluminum sheets coated with silica gel 60 (Merck 5554). Melting points were determined on a hotplate melting point apparatus in an open-mouth capillary and are uncorrected. The CHN analysis data for the structure **3** in this paper were recorded from IACS, Kolkata. DMSO for spectra detection was HPLC reagent grade without fluorescent impurities and H_2O was deionized water (Millipore).

Synthesis of 9-(butane-1-yl)-9*H*-carbazole-3,6-dicarbaldehyde (2)

Phosphoryl chloride (0.1 mol) was added dropwise into cooled, *N*-dimethylformamide (DMF, 0.1 mol) in an ice bath. The mixture was maintained at room temperature for 1 h and a solution of **1** (0.004 mol) in 5 mL of DMF was added. The reaction mixture was heated at 130 °C with stirring for 24 h and then poured into cracked ice. After neutralizing with a base, the mixture was extracted with chloroform. The extract was dried with anhydrous magnesium sulfate, and the solvent was removed by distillation in a vacuum. The solid residue was purified by using silica gel column chromatography (ethylacetate/hexanes) (1 : 4) to obtain a white solid (yield 62%). Mp 193 °C. ^{1}H NMR

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(300 MHz, $CDCl_3$): δ 10.07 (s, 2H), 8.61 (s, 2H), 8.04 (d, 2H, J = 7.14 Hz), 7.49 (d, 2H, J = 8.49 Hz), 4.33 (t, 2H, J = 7.2 Hz), 1.86 (m, 2H, J = 6.3 Hz), 1.40 (m, 2H), 0.91 (t, 3H, J = 1.11 Hz). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 141.01, 130.06, 125.54, 122.96, 120.07, 109.15, 52.44, 44.35, 32.44, 21.06, 14.25. Mass (LCMS): $(M + 1)^+$ 280.10.

Synthesis of 9-(butane-1-yl)-9*H*-carbazole-3,6-dihydrazinecarbothioamide (3)

To a solution of **2** (0.1 g, 0.358 mmol) in dry CH_3OH (60 mL) thiosemicarbazide (0.065 g, 0.716 mmol) was added. After refluxing at 70 °C for 12 h, the solvent was removed under reduced pressure giving the crude product. The product was recrystallized from hot methanol to give a pale yellow solid (0.13 g, 85% yield).

Mp > 250 °C. ^1H NMR (DMSO-d_6, 400 MHz): δ 11.4 (s, 2H, NH–C═S), 8.57 (s, 2H, CH_{imine}), 8.19 (d, J = 12 Hz, 2H, ArH), 7.94 (s, 2H, ArH), 7.62(d, J = 12 Hz, 2H, ArH), 6.38 (s, 4H, C═S–NH_2), 4.44 (t, J = 8 Hz, 3H, -NCH_2), 1.71 (m, 2H), 1.38 (m, 2H), 0.84 (t, J = 8 Hz, 3H, -CH_3). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 177.38, 144.09, 142.19, 126.41, 126.09, 123.05, 121.15, 111.27, 42.48, 31.44, 20.43, 14.39. ESI-MS: m/z $[M - 2]^+$: 423.1; calculated, 425.3. Anal. cald for $C_{20}H_{23}N_7S_2$ (425.3); C: 56.47; H: 5.40; N: 23.04; S,15.07. Found: C: 56.52; H: 5.31; N: 23.07; S: 15.01%.

Determination of binding constants

Binding constant values were determined by fluorescence and absorption methods using eqn (1) and 2.[19]

$$I = (I_0 + I\mathrm{K}C_G)/(1 + \mathrm{K}C_G) \quad (1)$$

$$I = I_0 + [(I - I_0)/2C_H] \\ (C_H + C_G + 1/\mathrm{K} - [(C_H + C_G + 1/\mathrm{K})^2 - 4C_HC_G]^{0.5} \quad (2)$$

where, I represent fluorescence intensity; I_0 represents the intensity of pure host; C_H and C_G are the corresponding concentrations of host and the guest; K is the association constant (this equation also works in absorption). The association constants and correlation coefficients (R), obtained by a nonlinear least-square analysis of I *vs.* C_G for eqn (1) and in case of eqn (2) the association constant and correlation coefficients (R), obtained by a non-linear least-square analysis of I *vs.* C_H and C_G.

Preparation of sample solutions for the evaluation of ion specificity

Stock solutions of 0.01 M of chloride of Na^+, K^+, Mg^{2+}, Ca^{2+}, Hg^{2+} and perchlorate of Co^{2+}, Pb^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Ni^{+2}, Cr^{+3}, Mn^{+2}, and Fe^{3+} were prepared in de-ionized water. Stock solutions of 0.01 M of anions are tetrabutylammoniums and potassium salts were prepared in pH 7.0 HEPES buffer (50 mM, pH 7.4, containing 1.0% DMSO). Stock solutions of the receptor **3** was prepared in H_2O containing 1.0% DMSO and 10 mM HEPES buffer pH = 7.0 in the concentration range ~ 10^{-6} M. 2.5 ml of the receptor solution was taken in the cuvette. Stock solutions of guests in the concentration range ~ 10^{-4} M, were prepared in the same solvents and were individually added in different amounts to the receptor solution. Upon addition of guests, the change in emission of the receptor was noted. The same stock solutions for receptors and guests were used to perform the UV-vis titration experiment. Guest solution was successively added in different amounts to the receptor solution (2.5 mL) taken in the cuvette and the absorption spectra were recorded. Both fluorescence and UV-vis titration experiments were carried out at 25 °C. All the experiments were repeated thrice to check the reproducibility.

The stoichiometry was determined by the continuous variation method (Job Plot). The concentration of the complex *i.e.*, [HG] was calculated using the equation [HG] = $\Delta I/I_0$ x [H] or [HG] = $\Delta A/A_0$ x [H] where $\Delta I/I_0$ and $\Delta A/A_0$ indicate the relative emission and absorbance intensities. [H] corresponds the concentration of pure host. Mole fraction of the host (X_H) was plotted against concentration of the complex [HG]. In the plot, the mole fraction of the host at which the concentration of the host–guest complex concentration [HG] is maximum, gives the stoichiometry of the complex.

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