CHAPTER 6

Benzimidazolium based dipod for quantification of iodide under physiological conditions, in tap water, common salt and live cell imaging

Abstract

Dipod 1 bearing two pyrenyl tethered benzimidazolium moieties gives fluorescence spectrum in aqueous medium which shows the structured emission band between 330-400 nm and a broad emission band centered at 480 nm, respectively due to monomer and excimer emission of pyrene moieties. Dipod 1 undergoes highly selective fluorescence quenching of the excimer emission band in the presence of iodide ions and can detect as low as 1 nM iodide in water and sea water conditions. Dipod 2, the perchlorate derivative of dipod 1, shows higher order of selectivity and sensitivity towards iodide ions with lowest detection limit of 0.5 nM. On using paper strips coated with dipod 1, 1.66 pg cm\(^{-2}\) iodide ions could be detected. Dipod 1 can determine 100 nM iodide ions in C6 glioma cells using confocal microscopy.

6.1 Introduction

Anions are important constituents of chemical and biological processes as majority of intracellular operations involve anionic species\(^1\). Consequently, the detection or sensing of biologically relevant anions under physiological conditions both outside and inside the cells has attained great significance and impetus\(^2\). Amongst anions, iodide has raised a special attention as it is one of the essential micronutrient for the normal growth of the human beings and 150 μg/day of its dose has been recommended\(^3\). In humans, the deficiency of dietary iodine or mal-functioning of thyroid proteins may cause goiter, hypothyroidism and hyperthyroidism. In humans, sodium iodide symporter and iodothyrosinase-deiodinase are important plasma membrane proteins to utilize the dietary iodide\(^4\).

According to world health organisation, if the iodide concentration in urine of a person or in a group of people is < 100 μg/L (< 8 nM), the person / group of people is considered to be iodine deficient. As in urine, > 90% of total iodine is present as iodide ion, the determination of iodide concentration becomes valuable for diagnosis and treatment of iodine deficiency. Also, for the diagnosis of transient thyroid dysfunction and iodine-induced hyperthyrosis, the measuring iodide concentration in urine becomes
Despite iodine being an essential trace element, it belongs to the category of toxic elements, especially because one of its radioactive isotope $^{129}$I is characterized by a long half-life ($1.6 \times 10^7$ years), as well as their tendency to weakly adsorb on minerals, making it able to reach the biosphere before decaying to significant levels. Therefore, we need rapid, sensitive and selective methods for the detection of iodide in food, biological samples and pharmaceutical products and for radioactive waste management to detect any leaching of iodine species from containers stored in geological sites.

Amongst halide ions, iodide ion has the weakest binding capacity with abiotic receptors owing to its large size and weaker basicity amongst all halide ions and makes it more challenging to develop receptors for iodide ions. The number of analytical approaches has been developed to detect iodide$^{6-12}$. One such approach employs displacement of metal ion especially Hg$^{2+}$ from non-fluorescent receptor-metal ion ‘ensemble’ and addition of iodide releases the receptor into the solution with the revival of the fluorescence$^{6,7}$. Surface enhanced Raman scattering (SERS)$^8$ has also been used for detection of iodide ions from environmental and food samples. Senapati et al have achieved desired sensitivity and selectivity by measuring the change in the SERS intensity$^8$ originating from Rh6G-adsorbed 30 nm gold nanoparticles upon the addition of iodide. In another approach gold or silver nano-particles along with organic molecules$^9$ have been used for detection of iodide ions. Amongst molecular receptors developed for recognition of iodide through supramolecular interactions, many of these probes are active in organic$^{10}$ or semi-aqueous$^{11}$ medium and find difficulty in determination of iodide in real situations.

Only a limited number of reports for iodide sensing by organic optical probes in aqueous media are available$^{12}$. Such organic optical probes interact with iodide ion through hydrogen bonding by H-bond donors viz. imidazolium C2-H or amid NH or boronic acid OH and undergo change in their optical properties, in aqueous medium. However, in these cases the detection limits achieved for the detection of iodide ions are quite high for their applications. Therefore, development of fluorescent sensors which can selectively bind with iodide ion in aqueous medium remains a challenge and such receptors’ applicability in live cell imaging has met with a very limited success$^7$.

We have reported number of probes, where the N-aryl benzimidazolium derivatives due to their ionic character remain soluble in water and due to N-aryl moieties create hydrophobic cavities suitable for selective interactions with variety of analytes. In such probes CH-anion interactions with ArCH and ArCH$_3$ moieties, though weak, but
contribute significantly for the recognition of ClO$_4^-$ and other anions$^{13}$. ClO$_4^-$ and I$^-$ have nearly similar charge densities and equal size and are well known to replace each other in the biochemical processes. Here in, we have designed a probe dipod 1 possessing two benzimidazolium groups as binding sites linked through durene spacer and having pyrene rings as fluorescent appendages. Dipod 1 in its fluorescence spectrum shows the presence of both pyrene monomer and excimer bands and has been proposed to attain equilibrium between open and closed structures. Dipod 1 shows highly selective quenching in fluorescence intensity only in the presence of iodide ions under physiological conditions and can be used to detect iodide ions in sea water conditions and tap water with lowest limit of detection as 1 nM. Dipod 1 also finds application for live cell imaging of iodide ions in C6 glioma cells.

6.2 Results and Discussion

6.2.1 Synthesis of Probe 1 and 2

Scheme 1: Synthesis of pyrenyl tethered benzimidazolium based dipods 1 and 2.

Dipod 1 was synthesized using protocol as shown in scheme 1. The arylation of benzimidazole with 4-bromophenol under CuI-benzotriazole catalyzed reaction conditions gave 1-(4-hydroxyphenyl)benzimidazole (4) in 75% yield. The compound 4
on O-alkylation with 1,4-bis(bromomethyl)-2,3,5,6-tetramethyl-benzene (5) gave 6, 70 %, m.p. 210 °C, HRMS 579.2640 (M+H)^+. The fluorescent pyrene units were then tethered via reaction of 6 with 1-bromomethylpyrene in CH$_3$CN under reflux for 24h to afford 1, 60 %, m.p. 280 °C, HRMS 1089.1441 (calculated for C$_{72}$H$_{56}$BrN$_4$O$_2$^+, 1089.2881). The $^1$H NMR spectrum shows the presence of two 4H singlets at δ 5.24 and 6.66, respectively due to OCH$_2$ and NCH$_2$ protons and benzimidazolium C2-H singlet (2H) at δ 10.22 and confirms the formation of dipod 1. The anion exchange of dipod 1 with NaClO$_4$ gave dipod 2.

6.2.2 UV-VIS and Fluorescence studies of dipod 1

The electronic spectrum of dipod 1 (5 µM, CH$_3$CN) exhibits three absorption bands at 312, 328 and 344 nm, typical of pyrene moiety. However, in HEPES buffer (5% DMSO, pH 7.4), the absorption intensity is decreased and absorption bands are red-shifted to 336 and 348 nm (Figure 1a). On excitation at 330 nm, the acetonitrile solution of dipod 1 exhibits emission spectrum with three maxima at 376, 397 and 418 due to pyrene moiety along with a small emission between 450-600 nm. The emission spectrum of dipod 1 in buffer reveals enhanced emission with maxima 376, 397 and 417 nm assigned to monomer emission and a structure less emission band centered at 480 nm due to excimer band of pyrene moieties (Figure 1b). Therefore, in the emission spectrum of dipod 1 in acetonitrile, the presence of emission band only due to monomer and in buffer the presence of both monomer and excimer emission bands elicit that dipod 1 in acetonitrile remains mainly in open form and in buffer exists as a mixture of both closed and open structures (Figure 2).

![Figure 1: (a) UV-Visible and (b) emission spectra of dipod 1 (5 µM) in acetonitrile and HEPES buffer (5% DMSO).](image-url)
In the preliminary experiments, the effect of various anions viz. F⁻, Cl⁻, Br⁻, CN⁻, SCN⁻, SO₄²⁻, ClO₄⁻, PO₄³⁻, NO₃⁻, AcO⁻, OH⁻ (25 µM each) on the UV-VIS and fluorescence spectra of dipod 1 (5 µM) was studied. On addition of these anions, the UV-VIS spectrum of dipod 1 did not show any significant change in the absorption spectrum. The fluorescence spectrum of dipod 1, on addition of iodide ions revealed decrease (80%) in fluorescence intensity of the excimer emission band at 480 nm (Figure 3), but the structured emission band centered at 395 nm remained unaffected. On addition of ClO₄⁻, the emission intensity of excimer band at 480 nm was enhanced (~40%) but the structured emission band centered at 395 nm did not show any change. The addition of other anions had insignificant effect on the fluorescence intensity of dipod 1.

On gradual addition of aliquots of KI, the fluorescence spectrum of solution of dipod 1 (5 µM) underwent gradual decrease in fluorescence intensity of the excimer band between 0 – 1 µM (Figure 4) and then slowly achieved the plateau. The fluorescence
quantum yield\textsuperscript{14} of the excimer band of dipod 1 on addition of iodide decreased from 0.015 to 0.004. Significantly, the fluorescence intensity of structured emission band due to monomer emission of pyrene centered at 395 nm remained unchanged even on addition of excess of iodide ions. The plot of fluorescence intensity vs I concentration does not show linear change and does not follow Stern-Volmer equation \( I/I_0 = A + K\theta Q \). The plot of \( I/I_0 \) vs \( \log [\Gamma] \) follows linear change \( (R^2 = 0.9976) \) (Figure 4c) between 5 nM to 5000 nM (5 µM). Consequently, Stern-Volmer constant \( (K_{SV}) \) has been determined by using exponential equation \( (I/I_0 = Ae^{K_{SV}Q} + B) \) and is found to be \( 3.7 \times 10^5 \) M\textsuperscript{-1}. The lowest limit of detection for I is 5 nM as evaluated on the basis of the signal-to-noise ratio of three\textsuperscript{15}. It is quite significant that nearly 50% of the fluorescence quenching is achieved on addition of 150 nM iodide ions to the 5 µM solution of dipod 1.

Figure 4: (a) Effect of addition of I on the fluorescence spectrum of 1 (5 µM, HEPES buffer - 5% DMSO, pH 7.4), \( \lambda_{ex} = 330 \) nm. (b) Plot of \( I/I_0 \) vs [I] (c) plot of \( I/I_0 \) Vs log [I]

Figure 5: (a) Job’s Plot of dipod 1 with KI indicating mixture of 1:1 and 2:1 stoichiometry of dipod 1 with iodide ions. (b) Distribution of different species on addition of KI to solution of dipod 1 as determined by regression analysis of fluorescence titration data

The spectral fitting of these data using non-linear regression analysis shows the formation of mixture of 1:1 and 2:1 stoichiometric (dipod 1: I) complexes with binding constant \( \log \beta_{L(I)} = 6.61 \pm 0.08 \) (L:I, 1:1) and \( \log \beta_{L_2(I)} = 13.17 \pm 0.14 \) (L:I, 2:1). The
Job’s plot (Figure 5a) shows inflection point at 0.4 mole fraction with an unsymmetrical peak shape and suggests the formation of 3:2; or mixture of 1:1 and 2:1 dipod 1:Γ stoichiometric complexes. The specfit results justify the observed stoichiometry from Job’s plot (Figure 5b). Therefore, dipod 1 forms mixture of 1:1 and 2:1 (dipod 1 : iodide) stoichiometric complexes.

Figure 6: A) The effect of gradual addition of iodide ions on the fluorescence spectrum of dipod 2 (HEPES buffer- 5% DMSO pH 7.4). λex = 330 nm.

On gradual addition of iodide ions to the solution of dipod 2, the fluorescence intensity of excimer emission band at 480 nm was gradually decreased up to 10 µM of iodide ions and then plateau was achieved. The fluorescence intensity of monomer emission band remained constant (Figure 6). The spectral fitting of these data by non linear regression analysis reveals the formation of mixture of 1:1 and 2:1 (dipod 2 : Γ) stoichiometric complexes. The association constants for complexation of dipod 2 with iodide ions were found to be log β_{L(Γ)} = 7.02 ± 0.07 (L:Γ, 1:1) and log β_{L2(Γ)} = 11.94 ± 0.05 (L:Γ, 2:1). Consequently, Stern-Volmer constant (KSV) has been determined by using exponential equation (I/I₀=Ae^{KSV}[Q] + B) and is found to be 3.5x10⁶ M⁻¹. The plot of I/I₀ vs log [Γ] follows linear change (R² = 0.9892) (Figure 6b) between 0.5 nM to 20000 nM (20 µM). Therefore, dipod 2 elicits the large range for linear change in fluorescence intensity with iodide ions i.e. over five orders of iodide concentration. The lowest limit of detection for Γ is 0.5 nM as evaluated on the basis of the signal-to-noise ratio of three⁴⁵.

6.2.3 Interference studies of dipod 1

The competition experiments with different anions at their elevated concentrations were performed to ascertain the selectivity of dipod 1 for iodide ions. It was observed
that the fluorescence intensity of 1 (5 µM) at 480 nm on addition of 50 µM of other interfering anions viz. F\(^-\), Cl\(^-\), Br\(^-\), CN\(^-\), SCN\(^-\), SO\(_4\)^{2-}, PO\(_4\)^{2-}, NO\(_3\)^{2-}, AcO\(^-\), OH\(^-\) and then on addition of 5 µM of iodide ions showed same change in the presence or absence of interfering anions (Figure 7). On using dipod 2, the interference of even perchlorate ions was not observed. Therefore, dipods 1 and 2 can be used as general probes for the estimation of iodide ions using fluorescence intensity as output.

**Figure 7:** Variation of fluorescence intensity at 480 nm for complex between dipod 1 (5 µM) and iodide (5 µM) in the presence of different anions (50 µM) in pH 7.4 HEPES buffer - 5% DMSO (\(\lambda\)\(_{ex}\) = 330 nm)

### 6.2.4 Mechanism of interaction

\(^1\)H NMR spectrum of dipod 1 in DMSO-\(d_6\) – water (9:1) on addition of iodide ions shows up-field shift of benzimidazolium C2-H by \(\delta\) 0.13. The other signals due to aromatic protons are also shifted up-field but to a smaller extent (< 0.07\(\delta\)). Probably, the dipod remains solvated in polar DMSO-water medium but in the presence of iodide ions, the formation of 2:1 stoichiometric complex with iodide ion (as evident from Job’s plot and regression analysis) results in decreased interaction with polar solvent molecules. This results in up-field shift of the proton signals. Therefore, dipod 1 interacts with iodide ion in the ground state.

In order to ascertain the mechanism of quenching, the life time of the free probe and on addition of various concentrations of iodide ions was determined. No-change in life time on addition of stimuli points to the static mechanism whereas change in life time corresponds to dynamic mechanism. The solution of dipod 1 on excitation with 345 nm laser and curve fitting of the data obtained shows the presence of two species with life
time 2.94 ns and 16.6 ns. Life time of both components decreased gradually on gradual addition of iodide ions. Plot of life time of these components against log [I−] shows linear change (Figure 8), which is parallel with above discussed linear change in fluorescence intensity of dipod 1 against log [I−]. These results clearly elicit that dynamic quenching process has the major contribution to the fluorescence quenching of dipod 1. These results also reveal that the H---I iodo-hydrogen bond of iodide ion with benzimidazolium C2-H units could be the major mode of recognition of iodide ions and the contribution of heavy atom effect on fluorescence quenching, if at all, is quite small.

**Figure 8**: Linear change in life time (ns) against log[iodide] (µM) (a) longer life time and (b) shorter life time components

### 6.2.5 Determination of iodide in real samples

In order to develop the applications of dipod 1 in real samples, the samples of tap water, solution of sodium chloride (0.6 M equal to concentration in sea water), and blood serum spiked with iodide ions were studied.

The distilled water in water-DMSO (95:5) mixture was replaced by tap water. The concentrations of iodide ions (5 – 5000 nM) were added to different solutions and their fluorescence intensity was measured. The concentration of iodide ions was determined from calibration curve recorded for the estimation of iodide ions under standard conditions. Table 1 reveals that the maximum relative standard deviation is ≤ 1.70% and pooled relative SD (PRSD) is 1.46% and maximum relative error is ≤ 1.3% and pooled relative error (PRE) is 1.08%.

LR grade sodium chloride was dissolved in Millipore water to prepare 0.6 M solution which is equivalent to concentration of sodium chloride in sea water. Stock solution of dipod 1 (15 µL) diluted with DMSO (135 µL) and was diluted to
3 ml with 0.6 M NaCl solution. Specific amounts of KI solutions were added and their fluorescence spectra were recorded. The concentration of iodide ions in each solution was determined from calibration curve recorded for the estimation of iodide ions under standard conditions. The iodide ions could be determined (table 1) with high precision and accuracy.

**Table 1**: Application of dipod 1 in determination of iodide ions in tap water, in the presence of NaCl and in blood serum samples

<table>
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<th>S. No.</th>
<th>Conc. ClO₄⁻ (nM)</th>
<th>Conc. determined ± SD (nM)</th>
<th>Relative error (%)</th>
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<td>Maximum relative error ≤ 1.30 and PRE = 1.08</td>
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<td>50</td>
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<td>0.9</td>
</tr>
<tr>
<td>4</td>
<td>500</td>
<td>501.5 ± 8.02</td>
<td>1.20</td>
</tr>
<tr>
<td>5</td>
<td>5000</td>
<td>5005 ± 66.4</td>
<td>1.01</td>
</tr>
<tr>
<td>In presence of NaCl</td>
<td>Maximum relative standard deviation ≤ 1.82 and PRSD = 1.38</td>
<td>Maximum relative error ≤ 1.41 and PRE = 1.02</td>
<td></td>
</tr>
<tr>
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<td>0.52 ± 0.006</td>
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<tr>
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<tr>
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<td>0.84</td>
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</table>

**6.2.6 Bioimaging of iodide ions in live C6 glioma cells**

![Figure 9: MTT assay of Dipod 1 and KI](image)
To demonstrate the applications of dipod 1 in live cell imaging, the glial cells of the rat brain (C6 glioma cells) were incubated with dipod 1. The MTT assay with C6 cells shows no significant difference in the proliferation of the cells in the absence or presence of 5–50 μM of dipod 1 (Figure 9). Therefore, dipod 1 has very low toxicity towards C6 glioma cell lines. C6 glioma cells themselves and after incubation with dipod 1 (10 μM) (30 min), on excitation with 405 nm laser exhibited fluorescence emission (Figure 10B). These cells pre-treated with dipod 1 (10 μM) were loaded with iodide ions (100 nM and 1 μM) solutions for half an hour. The cells pre-treated with dipod 1 and then loaded with 100 nM iodide ions show significant fluorescence quenching both in the blue and green windows of the detector (Figure 10C). The increase in the concentration of iodide ions from 100 nM to 10 μM caused almost total fluorescence quenching (Figure 10D). Fluorescent green or blue signals were visible only in the perinuclear region of the cytosol. Therefore, dipod 1 is permeable to C6 cells and can be used for imaging of as low as 100 nM iodide ions. The permeation of dipod 1 probably does not take place through the nuclear membrane and as a result the nuclear region gave no fluorescence after incubation with dipod 1 alone or in combination with 100 nM - 1 μM iodide ions.
Figure 10: Respective images of C6 glioma cells are as brightfield image and under blue window and green window (A) image of untreated C6 glioma cells, (B) fluorescence image of C6 glioma cells treated with dipod-1, (c,d) fluorescence image of C6 glioma cells treated with dipod 1 and then with 100 nM and 1 μM I, respectively.

There are only couple of examples in literature, where live cell imaging of iodide ions has been studied. In both the cases, bis-(thiosemicarbazone) derivatives were internalized in the live cells which underwent fluorescence quenching on incubation with Hg$^{2+}$ ions (1.2 and 10 μM), ascribed to the formation of probe-Hg$^{2+}$ ensemble. This ensemble underwent displacement reaction with iodide ions to release the fluorescence of bis-(thiosemicarbazone).derivative The present work provides the first example where the non-covalent interaction of iodide ions with the fluorescent probe has been used for imaging of iodide ions in live cells.

6.2.7 Contact mode method for detection of Iodide

The change in the fluorescence or colour of the paper strip on addition of an analyte provides a simple and cost effective method for qualitative determination of the analyte. To elaborate the application of dipod 1, we have carried out experiments based on contact mode recognition of iodide ions on paper strips made by dipping into aqueous solution of dipod 1 followed by drying in the incubator at 25°C. The different concentrations of iodide ions were prepared in aqueous solution followed by addition of aliquot of 10 μL of each solution on the paper strips previously coated with 1 (5 μM). For control experiment, drop of water alone was added on the paper strip coated with 1. The paper strips were then visualized under 365 nm UV light. We observed that the addition of 10 μL of $10^{-9}$ M I ion causes naked eye observable quenching of the fluorescence intensity under illumination of 365 nm light (Figure 11). The complete quenching of fluorescence intensity was observed with $10^{-6}$ M KI (Figure 11f). Figure 11A shows no quenching of fluorescence with only water. Therefore, the minimum amount of iodide detectable was up to the 1.66 pg/cm$^2$ under illumination at 365 nm.

Figure 11. Photographs of fluorescence quenching (under 365 nm UV light) of dipod 1 coated paper strips for the detection of iodide ions; (A) paper strip with a drop of water; Iodide ions of
different concentration (B) $10^{-9}$ M (C) $10^{-8}$ M (D) $10^{-7}$ M (E) $10^{-6}$ M. [The size of each paper strip is 1cm$^2$].

6.3 Conclusions

In conclusion, we have designed and synthesized a pyrenyl tethered benzimidazolium based fluorescent dipod 1 for the selective detection of iodide ions in HEPES buffer. Dipod 1 in aqueous medium gives fluorescence spectrum which shows the structured emission band between 330-400 nm and a broad emission band centered at 480 nm, respectively due to monomer and excimer emission of pyrene moieties. Dipod 1 underwent fluorescence quenching of excimer emission band only in the presence of iodide ions amongst number of other anions studied. dipods 1 and 2 elicited linear change in fluorescence intensity vs log[I$^{-}$] over a broad pH range between $10^{-9}$ to $10^{-5}$ M iodide solutions with limits of detection 5 nM and 0.5 nM, respectively. Dipod 1 could be used for estimation of iodide ions in real samples viz. tap water, sodium chloride and blood serum. Dipod 1 also could be used for live cell imaging of iodide ions in C6 glioma cells.

6.4 Experimental Section

6.4.1 General Procedures

Chemicals and solvents were reagent grade and used without further purification unless otherwise stated. Moisture-sensitive reactions were performed under N$_2$ atmosphere. Melting points were determined in open capillaries and are uncorrected. Chromatographic purification was done with silica gel 60-120 and 230-400 mesh ASTM. For monitoring the progress of the reaction and for comparison with authentic samples, thin layer chromatography (tlc) was used. For this purpose, micro slides were coated with silica gel 'G’ containing calcium sulfate as binder or with the silica gel HF-254 (Qualigens India), by dipping a pair of slides held back to back in slurry of adsorbent in chloroform-methanol (80:20). The chromatograms were developed in iodine chamber or with UV-254 lamp.

NMR spectra were recorded on Bruker-500 MHz machine. The peak values were obtained as ppm ($\delta$), and referenced to the TMS. Abbreviations used for splitting patterns are s = singlet, bs = broad singlet, t = triplet, q = quartet, m = multiplet. The fluorescence titrations were performed on Varian Carey Eclipse fluorescence spectrophotometer and
BH-CHRONOS spectrophotometers. The life-time studies were performed on BH-
CHRONOS spectrophotometer and absorption spectra were recorded on Shimadzu-2450
spectrophotometer.

All absorption and fluorescence scans were saved as ACS II files and further
processed in Excel™ to produce all graphs shown. The spectral data were analyzed
through curve fitting procedures by using non-linear regression analysis SPECFIT 3.0.36
to determine the stability constants and the distribution of various species.

6.4.2 Synthesis of 4-(1H-benzo[d]imidazol-1-yl)phenol (4): In a 100 ml two-neck RBF
CuI (0.11 g, 0.58 mmol) and benzotriazole (0.134 g, 1.12 mmol) were
dissolved in DMSO (5 mL) and stirred at RT under N₂. To this solution 4-bromophenol (1.9 g, 11.05
mmol), benzimidazole (0.89 g 7.5 mmol) and potassium tert-butoxide (1.6 g, 14.3 mmol)
were added and resulting solution was stirred at 110°C for 24 h. After completion of the
reaction, the mixture was treated with aqueous solution of EDTA and extracted with
ethyl acetate. The solvent was removed under vacuum and the crude mixture was column
chromatographed to isolate pure compound 4, white solid, 75% yield.

{H NMR (DMSO-d₆, 500 MHz): δ 6.98 (d, 2H, J = 8.5 Hz, ArH), 7.28 (t, 2H, J = 6.5 Hz, ArH), 7.43 (d, 2H, J = 8.5 Hz, ArH), 7.48 (d, 1H, J = 7.5 Hz, ArH), 7.75 (d, 1H, J = 8 Hz, ArH), 8.41
(s,1H, OH), 9.91 (s, 1H, Bim-C2H).}

13C NMR (DMSO-d₆, 125 MHz): δ 110.9, 116.8, 120.2, 122.6, 123.6, 126.0, 127.7, 134.2, 143.9, 157.6.

6.4.3 Synthesis of compound 6: To a solution of 4 (0.42 g, 2.0 mmol) in tetrahydrofuran,
KOH (0.45 g, 8.0 mmol) was added and the resulting mixture was stirred for 1h at RT.
1,4-Bis(bromomethyl)-2,3,5,6-tetramethylbenzene (0.32 g, 1.0 mmol) was added to the
solution and reaction mixture was stirred for additional 24 h. After completion of the
reaction, the crude mixture was extracted with ethyl acetate. The solvent was removed and the residue was column chromatographed to get compound 6, white solid; 70% yield, m.p. 210°C. HRMS m/z (TOF MS ES⁺) calculated for C₃₈H₃₄O₂N₄, 578.2682; found 579.2640 (M+nH); ^{1}H NMR (CDCl₃, 500 MHz): δ 2.40 (s, 12H, 4×CH₃) , 5.19 (s, 4H, 2×CH₂), 7.22 (d, 4H, J = 8.5 Hz, ArH ), 7.32-7.36 (m, 4H, ArH), 7.46-7.51 (m, 6H, ArH), 7.89 (d, 2H, J = 7.0 Hz, ArH), 8.08 (s, 2H, Bim-C2H). 13C NMR (DMSO-d₆, 125 MHz): δ 16.3, 65.8, 110.3, 115.8, 120.6, 122.6, 123.6, 125.8, 129.5, 133.1, 134.3, 134.8, 142.5, 143.9, 158.9.

6.4.4 Synthesis of Dipod 1: The solution of compound 6 (144.5 mg, 0.25 mmol) and 1-
bromomethylpyrene (155 mg, 0.525 mmol) in acetonitrile was refluxed for 24 h. The
white solid separated was found to be pure dipod 1, 63 %, m.p. 280°C; HRMS m/z (TOF MS ES+) calculated for C_{72}H_{56}BrN_{4}O_{2}^+, 1089.1441; found 1089.2881. \textsuperscript{1}H NMR (DMSO-d_{6}, 400 MHz): δ 2.50 (s, 12H, 4×CH_{3}), 5.24 (s, 4H, 2×OCH_{2}), 6.66 (s, 4H, 2×CH_{2}), 7.39 (d, 4H, ArH, J = 6 Hz), 7.72-7.74 (m, 4H, ArH), 7.80-7.86 (m, 6H, ArH), 8.08-8.44 (m, 18H, ArH), 8.66 (d, 2H, ArH, J = 6 Hz), 10.22 (s, 2H, Bim-C2H).

6.4.5 Bioimaging of iodide ions in live C6 glioma cells

C6 glioma cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 IU/ml penicillin, 100 µg/ml gentamycin and 100 µg/ml streptomycin. These cells were maintained in a humidified environment in an incubator at 37 °C with 5% CO_{2}. One day before treatment, a total of 2×10^5 cells were seeded on to 11 mm glass cover slips into each well of a 24-well plate, and these were grown for 24 h (until 60–70% confluence) and treatment with dipod 1 was carried out in triplicates in FBS and antibiotic free media (98% media supplemented with 2% DMSO). C6 cells were incubated with dipod 1 (5 µM) at 37 °C with 5% CO_{2} for 30 min followed by three times wash with 1X phosphate buffered saline (PBS) (pH=7.4) and then treated with cyanide ions (10 nM and 100 nM) for another half an hour by incubating the cells at the same conditions. The cells were then washed three times with 1X PBS (supplemented with 2% DMSO), fixed in ice cold 4% paraformaldehyde. Washed again three times with 1X PBS (supplemented with 2% DMSO), and mounted on glass slides. To investigate the cell proliferation and to test their viability with dipod 1, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay with C6 cell lines was also carried out. Cell imaging was carried out using Plan Apo 60X oil immersion objective lens.

6.4.6 UV-Vis and Fluorescence Titrations

The concentration of HEPES buffer (pH 7.04) was 0.05 M. Stock solutions of dipod 1 and dipod 2 (1 mM) were prepared in DMSO. For experiments with dipod 1 and dipod 2, we have taken 3 ml of the solution that contains 15 µL (60 µL) dipod 1 / dipod 2 (for 5 and 20 µM) solution in DMSO, 135 µL (90 µL) of DMSO and 2.85 ml of HEPES buffer (0.05 M, pH = 7.4) in cuvette. Typically, aliquots of freshly prepared standard solutions (10^{-1} M to 10^{-3} M) of sodium salts ( NaX), where X = CN⁻, F⁻, Cl⁻, Br⁻, I⁻, ClO_{4}⁻, NO_{3}⁻, SO_{4}^{2-}, HSO_{4}⁻, SCN⁻, AcO⁻ and
H$_2$PO$_4^-$ in deionized millipore water were used to record UV-VIS and fluorescence spectra.

6.4.7 Detection limit

The detection limit was calculated based on the fluorescence titration. To determine the S/N ratio, the emission intensity of dipod 1 (20 μM) and dipod 2 (5 μM) was measured 5 times and the standard deviation of blank measurements was determined. Under the present conditions, a good linear relationship between the fluorescence intensity and log [iodide] could be obtained in 5x10$^{-9}$ – 10$^{-5}$ M ($R^2 = 0.9976$) for dipod 1 and 0-10 nM ($R^2 = 0.9892$) for dipod 2. The detection limit was then calculated with the equation:

Detection limit = 3σbi/m

Where, σbi is the standard deviation of blank measurements; m is the slope between intensity versus sample concentration. The detection limit was measured to be 1 nM at S/N = 3.
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