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PAPER

FRET-based ratiometric detection of Hg^{2+} and biothiols using naphthalimide–rhodamine dyads†

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A new naphthalimide–rhodamine-based dyad **1** in CH_3CN–HEPES (1 : 1) buffer solution exhibits fluorescence resonance energy transfer (FRET) from naphthalimide to the rhodamine moiety on addition of only Hg^{2+} ions and allows ratiometric absorption and fluorimetric estimation of Hg^{2+} ions between 50 nM (10 ppb) to 2 μM (0.4 ppm). FRET-induced fluorescence changes were recovered again by the subsequent addition of thiol amino acids *via* reverse FRET. The interconversion of probe **1** and **1**·Hg^{2+} *via* the complexation/decomplexation by the modulation of Hg^{2+}/Cys exhibited a selective probe for biothiols in real samples.

1. Introduction

Fluorometry is becoming important for ion sensing because of its simplicity, high selectivity and sensitivity.[1] Most fluorometric sensors are designed to adopt photophysical changes produced upon complexation, including photoinduced electron transfer (PET),[2] photoinduced charge transfer (PCT),[3] excimer/exciplex formation and extinction,[4] or fluorescence resonance energy transfer (FRET).[5] FRET-based probes are preferred over single dye-based probes, as the FRET process can quantify the analyte concentration by using the ratio of intensities of the well resolved fluorescence peaks with reasonable intensities at two different wavelengths for free probe and analyte-bound probe.[6] Recently, FRET-based systems[7] have actively progressed due to their practical benefits in cell physiology and optical therapy, as well as selective and sensitive sensing toward targeted analytes.[8] However, despite numerous advantages, only very scarce examples of FRET-based probes for ratiometric sensing of Hg^{2+} [9] and biothiols have been reported.

Mercury undergoes long-range transport in the environment among various media by deposition from anthropogenic sources, including oceanic and volcanic emissions, gold mining and combustion of fossil fuels.[10] The biological targets and toxicity profile of mercury species[11] depend on their chemical composition.[12] The exposure to mercury, even at very low concentrations, leads to digestive, kidney and especially neurological diseases,[13] as mercury can easily pass through the biological membranes. Thus, keeping in view the role played by mercury in day-to-day life, simple and rapid sensing of mercury[14] in biological and environmental systems is very important. Biothiols, such as cysteine (Cys), homocysteine (Hcy) and glutathione (GSH), also play crucial roles in many physiological processes. Alterations in the level of cellular thiols have been linked to a number of diseases, such as cardiovascular disease, Alzheimer's disease, leucocyte loss, psoriasis, liver damage, cancer and AIDS.[15] Thus, it is important to develop efficient methods for the detection and quantification of biothiols in physiological media.[16] Currently, a number of thiol-reactive fluorescent probes based on different mechanisms have been reported in the literature.[17] But it is highly desirable to develop reversible fluorescent probes for the sensitive and selective determination of mercapto biomolecules.[18f]

Herein, we have developed a new FRET-based rhodamine–naphthalimide dyad **1**, composed of rhodamine and naphthalimide moieties. Rhodamine B in open form has strong absorption at around 525 nm, whereas the spirocyclic ring form has absorption only in the UV region. A naphthalimide derivative (450 nm) has been chosen as the donor, which has a significant difference in spectral overlap integral between the two forms of rhodamine B and can be independently excited. In the case of dyad **1**, rhodamine exists in spirocyclic ring form and has no absorption band in the wavelength region of naphthalimide emission so there is no overlap between naphthalimide emission and rhodamine absorption. Thus, the rhodamine moiety cannot accept the excitation energy of the donor moiety, and the donor fluorescence (485 nm) is observed and FRET remains "off". Upon the addition of Hg^{2+}, the rhodamine spirocyclic ring opened, which has strong absorption (525 nm) in the naphthalimide emission region (485 nm). Then, rhodamine fluorescence (585 nm) due to FRET is observed. Thus, the Hg^{2+}-induced process changed the

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†Electronic supplementary information (ESI) available: Spectroscopic data for Probe **1** including ^1H and ^{13}C NMR spectra and mass spectrum, UV-Vis and fluorescence emission spectra of **1**. See DOI: 10.1039/c2ob25794g

emission maxima of the system from 485 nm (naphthalimide emission) to 585 nm (rhodamine emission). This red shift wavelength allowed the ratiometric detection of Hg^{2+} ion in an aqueous system. The emission changes obtained by the Hg^{2+} ions were recovered again by the addition of biothiols (Cys, Hcy, GSH) *via* reverse FRET phenomenon. Thus, probe **1** behaved as a selective chemosensor for Hg^{2+} ions and its inter-convertible fluorescent probe **1·Hg^{2+}** exhibited high sensitivity and selectivity to mercapto amino acids *via* a reversible decomplexation ratiometrically. According to the best of our knowledge, this is the first example in the literature for the ratiometric detection of biothiols in real samples based on a mercury ensemble system.

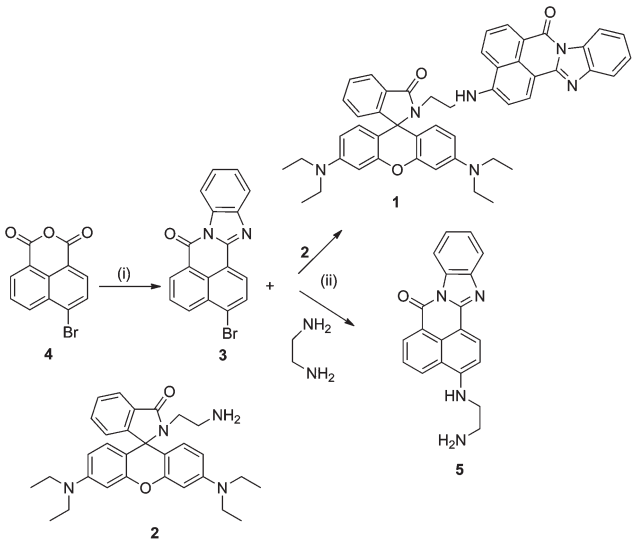
Results and discussion

Synthesis of probe 1

The dyad **1** was synthesized[19] on the basis of the route shown in Scheme 1. Oxidative coupling of **4** with 1,2-diaminobenzene in THF gave compound **3**, which on reaction with rhodamine ethylene-diamine gave dyad **1** in 65% yield. ^{1}H NMR, ^{13}C NMR and HRMS spectral data (S9–S13†) confirm the structure of dyad **1**.

For an efficient FRET phenomenon, the overlap of the absorption spectrum of the acceptor with the emission spectrum of the donor, efficiency of energy transfer from donor to acceptor, donor–acceptor distance and relative orientation of the donor and acceptor transition dipoles, are essentially required. The absorption spectrum of the open spirocycle form of the rhodamine B develops a significant overlap ($J_{DA} = 3.8 \times 10^{13}$ M^{-1} cm^{-1}) with the emission spectrum of **5** and clearly offers the possibility of a FRET process (Fig. 1). The efficiency of the energy transfer (ηEET) was evaluated using the equation[9b] $\eta EET = 1 - \Phi_{F(\text{donor in dyad 1})}/\Phi_{F(\text{free donor})}$. Here, $\Phi_{F(\text{donor in dyad})}$ is the fluorescence quantum yield of the donor part in dyad **1** and $\Phi_{F(\text{free donor})}$ is the fluorescence quantum yield of the donor when not connected to the acceptor. The fluorescence quantum yield of donor **5** in CH_3CN–HEPES buffer was 0.215, whereas the quantum yield of the naphthalimide part in dyad **1** is 0.05. Thus, the efficiency of naphthalimide (ηEET) in the 'on' state is very high *i.e.* up to 76.7%. Therefore, dyad **1** meets the criteria for the FRET mechanism. It is worth noting that the addition of Hg^{2+} ions to a solution of **5** caused fluorescence quenching only, no red shifted fluorescence band was observed, whereas **2** does not show any absorption and fluorescence changes on the addition of Hg^{2+} ions, which clearly depicted the FRET phenomenon from naphthalimide to rhodamine (closed form) in dyad **1** (Fig. S14†).

The absorption spectrum of dyad **1** (20 μM, CH_3CN–HEPES buffer, 1 : 1, pH = 7.12) showed an absorption maximum at 450 nm (ε = 21 000), predominantly due to an intra-ligand charge transfer (CT) transition in the naphthalimide moiety. On addition of metal cations *viz.* Hg^{2+}, Fe^{2+}, Fe^{3+}, Pb^{2+}, Cd^{2+}, Cu^{2+}, Zn^{2+}, Ni^{2+}, Ag^{+}, Co^{2+}, Mg^{2+}, Li^{+}, Na^{+} and K^{+} *etc.* as their perchlorate salts, a significant change in the electronic spectrum was observed only with Hg^{2+} ions (ESI†, Fig. S1). A new absorption band around 510 nm appeared with a detectable change in solution color from yellow to pink. The Cu^{2+} ions also showed a small color change from yellow to light pink, but only upon addition of 200 μM (10 equivalents) of Cu^{2+}.



Scheme 1

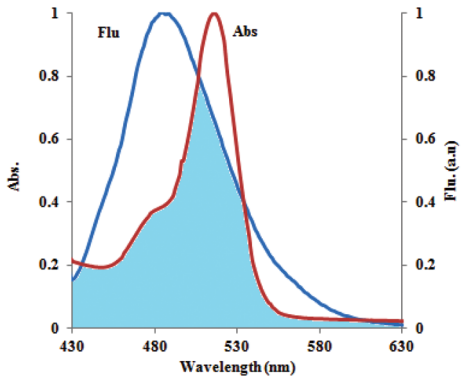


Fig. 1 The overlap (shown with cyan shade) between the emission and absorption spectra of the donor (naphthalimide) and acceptor (rhodamine) moieties.

On gradual addition of Hg^{2+} ions to the solution of **1** 20 μM, CH_3CN-HEPES buffer (1 : 1), the absorbance at 450 nm underwent a gradual decrease in its intensity with a concomitant increase in absorbance intensity at 510 nm, associated with two isosbestic points at 412 nm and 473 nm. This 60 nm red shift in the absorbance maxima points to the rhodamine spirolactam ring opening through interaction of **1** with Hg^{2+} ions (Fig. 2a).

The "OFF–ON" switching behavior on addition of Hg^{2+} ions to the solution of **1** at 450 nm and 510 nm provides dual absorption channels for elaborating a ratiometric approach, which permits signal rationing and allows the estimation of analyte independent of the concentration of the receptor. The ratios of absorption intensities at 510 and 450 nm (I_{510}/I_{450}) exhibit a drastic change from 0.16 in the absence of Hg^{2+} to 3.82 in the presence of 20 μM Hg^{2+} (Fig. 2b), a 23.8-fold variation in the absorption ratios. The dyad **1** (CH_3CN–HEPES buffer, 1 : 1) can be used for estimation of Hg^{2+} ions between 50 nM (10 ppb)–20 μM (0.4 ppm), (Fig. 2b). The spectral fitting of these absorbance data shows the formation of a 1 : 1 (**1** : Hg^{2+})

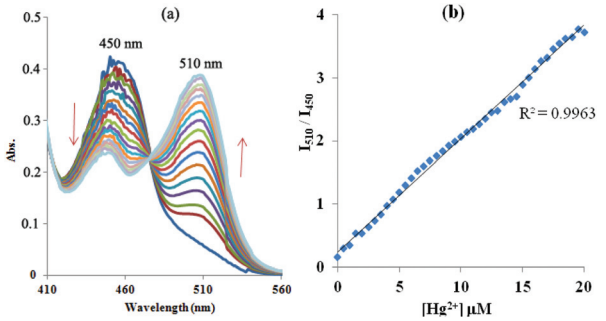


Fig. 2 The effect of the addition of Hg^{2+} ions on (a) the UV-Vis spectrum of **1** (20 μM, CH_3CN–HEPES buffer (1 : 1), pH = 7.12 ± 0.1). (b) Absorbance ratiometric (20 μM) responses (A_{510}/A_{450}) of dyad **1** upon incremental addition of Hg^{2+} ions showing the linear fit.

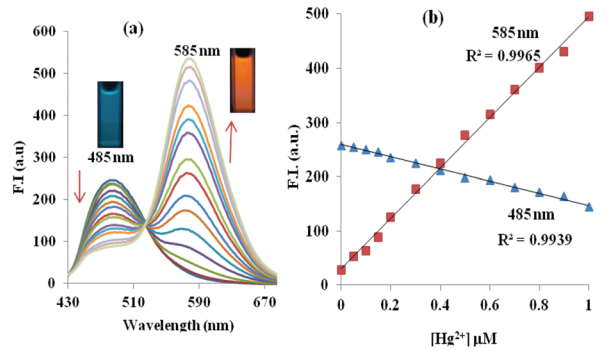
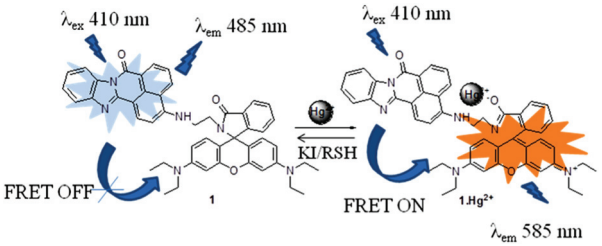


Fig. 3 (a) The effect of gradual addition of Hg^{2+} ions on the fluorescence spectrum of dyad **1** (1 μM, CH_3CN–HEPES buffer (1 : 1), pH = 7.12 ± 0.1). (b) The linear increase in the fluorescence intensity *vs.* $[Hg^{2+}]$ at both 485 nm and 585 nm.

stoichiometric complex [log $\beta_{LHg^{2+}}$ = 5.19 ± 0.12] and was also confirmed by Job's plot.

Upon excitation at 410 nm, the free dyad **1** displayed a single emission band centred at 485 nm (Φ = 0.21), attributed to the emission of naphthalimide. There was no FRET in the free **1**, as the rhodamine acceptor was in the ring-closed form. On addition of Hg^{2+} the fluorescence intensity at 485 nm significantly decreased with simultaneous appearance of a new red-shifted emission band at around 585 nm (Fig. 3a), ascribed to the emission of the rhodamine acceptor. This was also accompanied by a resonance color change from blue to red under 355 nm illumination of light. Also, the intensity of fluorescence emission at 485 nm and 585 nm, respectively decreased and increased linearly between 50 nM (10 ppb) and 1 μM (0.2 ppm) of Hg^{2+} ions (Fig. 3b). Thus, the addition of Hg^{2+} elicited a large red shift (100 nm) in emission, which almost completely resolved the two emission peaks. This fluorescence enhancement at 585 nm is typically attributed to the opening of the spirolactam ring of rhodamine-6G (Scheme 2).

The nonlinear regression analysis of the spectral data obtained on titration of the solution of **1** with Hg^{2+} ions shows the formation of 1 : 1 stoichiometric complex [log $\beta_{LHg^{2+}}$ = 6.22 ± 0.24]. The Job's plot analysis of the fluorescence titrations revealed a maximum at 0.5 mole fraction (Fig. 4b), also indicating 1 : 1 binding stoichiometry.



Scheme 2

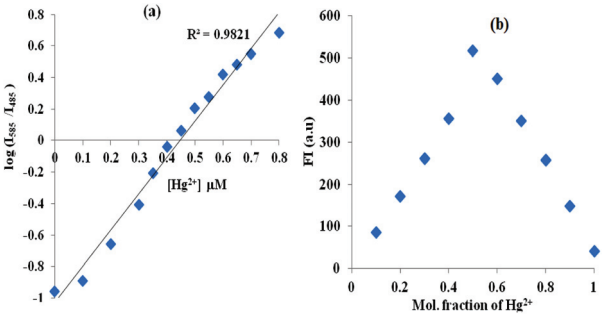


Fig. 4 (a) Fluorescence ratiometric response (F_{585}/A_{485}) of dyad **1** *versus* Hg^{2+} concentration. (b) A Job's plot showing the 1 : 1 stoichiometry between dyad **1** and Hg^{2+}.

The ratios of fluorescence intensities at 585 nm and 485 nm (I_{585}/I_{485}) exhibit a drastic change from 0.11 in the absence of Hg^{2+} to 15.6 in the presence of 1.0 μM Hg^{2+} (ESI†, Fig. S1–S2) – a 141-fold variation in the emission ratios. This ratio increases exponentially with an increase in concentration of Hg^{2+}. However, the plot of the log of the ratio of intensities shows an excellent linear relationship with concentrations of Hg^{2+} between 100–800 nM with R^2 = 0.9821 (Fig. 4a). The response of dyad **1** to Hg^{2+} was quite fast and all absorption and fluorescence spectra were recorded within 2 min of adding Hg^{2+}.

In order to prove the reversibility of the dyad **1**, the experiments were performed in the presence of KI. On addition of an aqueous solution of KI to the **1·Hg^{2+}** complex, the emission band at 585 nm disappeared with reappearance of the fluorescence maxima at 485 nm. Again, on addition of Hg^{2+} to this solution mixture, having KI, the emission band at 585 nm reappeared. Preferential binding of iodide ions to Hg^{2+} led to the formation of HgI_2 and the regeneration of the cyclic lactam form and confirms the reversible binding of rhodamine dyad to Hg^{2+} (Fig. 5).

We also tested the fluorescence response of **1** to other metal ions, such as Fe^{2+}, Fe^{3+}, Pb^{2+}, Cd^{2+}, Cu^{2+}, Zn^{2+}, Ni^{2+}, Ag^{+}, Co^{2+}, Mg^{2+}, Li^{+}, Na^{+} and K^{+}, in CH_3CN–HEPES buffer (1 : 1) and no significant variation in the fluorescence spectrum of **1** (Fig. 6) was observed with any other metal ion except Cu^{2+}, which induced emission at 585 nm, but to a very small extent. To check the practical ability of dyad **1** as a Hg^{2+}-selective fluorescent sensor, we carried out competitive experiments in the presence of 1 μM of Hg^{2+} and 100 μM of the interfering metal ions like Fe^{2+}, Fe^{3+}, Pb^{2+}, Cd^{2+}, Cu^{2+}, Zn^{2+}, Ni^{2+}, Ag^{+}, Co^{2+}, Mg^{2+}, Li^{+}, Na^{+} and K^{+}. As shown in Fig. 6, no significant variation in the fluorescence emission was observed by the addition of interfering metal ions.

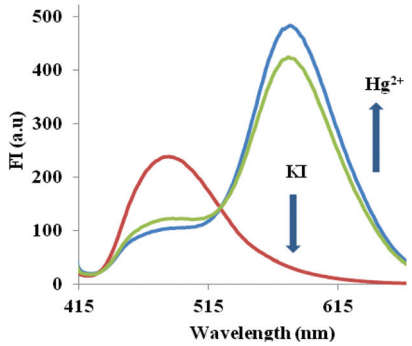


Fig. 5 Fluorescence spectra showing the reversibility of Hg^{2+} coordination to dyad **1** by KI: blue line, **1** + 1 μM Hg^{2+}; red line, **1** + 1 μM Hg^{2+}, +2 μM KI; green line, **1** + 1 μM Hg^{2+}, + 2 μM KI + 5 μM Hg^{2+}; in CH_3CN–H_2O (1 : 1), HEPES buffer (pH = 7.12 ± 0.1) at λ_{ex} = 410 nm.

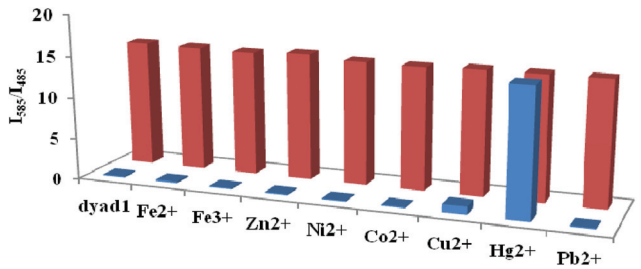


Fig. 6 Fluorescence ratiometric response (I_{585}/I_{485}) of **1** (1 μM) upon addition of different metal ions in CH_3CN–H_2O (1 : 1), HEPES buffer (pH = 7.0 ± 0.1). Blue bars show the effect of different metal ions on dyad **1** fluorescence intensity and red bars show the presence of interfering metal ions on dyad **1** + Hg^{2+} fluorescence intensity.

To investigate the ability of the **1·Hg^{2+}** complex as an ensemble probe for thiols in biological media, absorption and emission changes of an aqueous solution of **1·Hg^{2+}** in CH_3CN–HEPES buffer (1 : 1) were examined with the addition of various amino acids. The **1·Hg^{2+}** ensemble revealed remarkable emission changes with only the addition of thiol-containing amino acids, whereas no change was observed with other amino acids (Ala, Arg, Asp, Glu, Gly, His, Lys, Met, Pro, Ser, Thr, Trp, Tyr, Val) (Fig. 7a and S5–6†). It was found that the emission spectrum of a mixture of **1·Hg^{2+}** and thiol is identical to that of probe **1**. The incremental addition of mercapto biomolecules, such as Cys, Hcy or GSH, to the aqueous solution of **1·Hg^{2+}** resulted in revived UV-Vis absorption and fluorescence spectra of free probe **1** and also returned the fluorescence quantum yield to 0.3. As seen in Fig. 7b, when **1·Hg^{2+}** was titrated with Cys, the fluorescence intensity of the solution was proportional to the concentration of cysteine up to 2 equivalents. The detection limits estimated were 0.2 μM–2 μM for cysteine. Consistently, the visual emission color of **1·Hg^{2+}** solution turned from red to green as well. Among all of the amino acids tested, Cys gave the highest response toward the **1·Hg^{2+}** probe. As shown by Fig. 7a, fluorescence changes were highest at ~90% for Cys, 60% and 25% for Hcy and GSH, respectively, and below 5% for other amino acids.

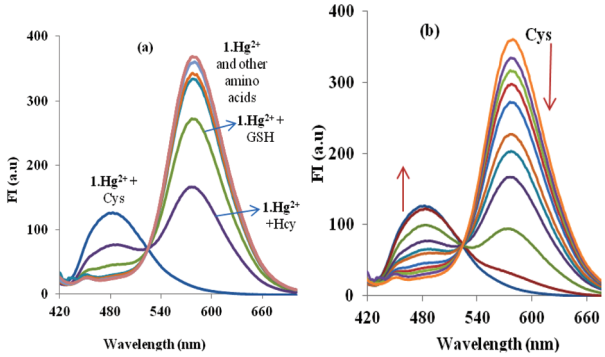


Fig. 7 (a) The effect of different amino acids on the fluorescence spectrum of **1·Hg^{2+}**. (b) The effect of incremental addition of cysteine on the fluorescence spectrum of the **1·Hg^{2+}** (1 : 1) complex at 1 μM concentration.

Interestingly, the alternate addition of a constant amount of Cys and Hg^{2+} to probe **1** gives rise to a switchable change in the fluorescence intensity at 585 nm. Such a reversible interconversion can be repeated more than 10 times by the modulation of Cys/Hg^{2+} added, indicating that probe **1** can be developed as a reversible fluorescence OFF–ON probe for Cys and Hg^{2+} (Fig. S6†).

To further show the potential utility of **1·Hg^{2+}** in biological samples, **1·Hg^{2+}** was further studied as a probe to detect biothiols in wheat samples, in which cysteine and other amino acids can be found free or linked to each other. It was confirmed that a trace amount of cysteine in a wheat sample can easily be detected using the **1·Hg^{2+}** probe. For the determination of biothiols, a wheat sample (*Triticum aestivium*, cv. PBW343) was first introduced to extract the different proteins *viz.* albumin, globulin, prolamin *etc.*, and then these proteins were hydrolysed to free amino acids using 6 N HCl (ESI†). The fluorescence spectral changes observed with hydrolysed proteins were similar, as observed with probe **1·Hg^{2+}** with cysteine (Fig. S8†). It shows that probe **1** can be used to selectively estimate thiols in the wheat sample.

Conclusions

We have designed and synthesized a FRET-based dyad **1** as a new chromogenic and fluorescent chemosensor suitable for ratiometric sensing of Hg^{2+} on the basis of the Hg^{2+}-induced "ON–OFF–ON" switching and ratiometric detection of biothiols based on FRET reversal. The well-resolved absorption and emission peaks, the high efficiency in energy transfer and large variation in the emission (I_{585}/I_{485}) ratios are the added advantages.

Experimental section

General

Synthesis of 3 and 4. Compounds **3** and **4** have been synthesized according to the literature method.[19]

Synthesis of 1. Rhodamine-6G-ethylenediamine **2** (242 mg, 0.5 mmol) was dissolved in 2-propanol and **3** (174 mg, 0.5 mmol) was added. The resulting solution was refluxed for 12 h. The progress of the reaction was monitored by TLC. After

completion of the reaction, the reaction mixture was cooled to room temperature and evaporated to dryness. The residue was purified through column chromatography to get pure dyad **1** in 65% yields as a yellow solid. Mpt 185 °C, ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.12 (t, J = 7.2 Hz, 12H, 4 × CH_3), 2.95 (t, J = 6.3 Hz, 2H, CH_2), 3.27 (q, J = 7.2 Hz, 8H, 4 × CH_2), 3.66 (t, J = 6.3 Hz, 2H, CH_2), 6.11 (d, J = 8.4 Hz, 1H, ArH), 6.22 (d, J = 8.4 Hz, 1H, ArH), 6.37–6.39 (m, 4H, ArH), 7.00–7.10 (m, 1H, ArH), 7.38–7.41 (m, 4H, ArH), 7.55 (s, 1H, ArH), 7.76 (t, J = 8.1 Hz, 1H, ArH), 7.83 (d, J = 8.4 Hz, 1H, ArH), 7.95 (t, J = 8.1 Hz, 1H, ArH), 8.34 (d, J = 8.4 Hz, 1H, ArH), 8.47 (d, J = 8.4 Hz, 1H, ArH), 8.58 (d, J = 8.4 Hz, 1H, ArH), 8.84 (d, J = 7.2 Hz, 1H, ArH). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 13.3, 41.6, 45.0, 57.8, 98.3, 106.0, 107.2, 108.6, 117.6, 119.0, 119.3, 123.5, 124.5, 128.7, 129.5, 130.2, 131.7, 131.8, 132.7, 133.1, 134.3, 135.2, 135.8, 149.4, 154.0, 161.7, 166.7, 170.2; HRMS (ESI): m/z, calcd 752.3475 (M^+); found 752.3321; elemental analysis: found C, 76.53; H, 5.92; N, 11.12%. $C_{48}H_{44}N_6O_3$ requires C, 76.57; H, 5.89; N, 11.16%.

Synthesis of 5. Compound **3** (348 mg, 1 mmol) was dissolved in 2-propanol and ethylene diamine (500 mg, 10 mmol) was added. The resulting solution was refluxed for 12 h. The progress of the reaction was monitored by TLC. After completion of the reaction, the reaction mixture was extracted with chloroform and evaporated to dryness. The residue was purified through column chromatography to get pure compound **5** in 80% yield as a creamish white solid. Mpt 170 °C: ^{1}H NMR (400 MHz, CDCl3) δ 8.55 (d, J = 6.44 Hz, 2H, ArH), 8.36 (d, J = 7.40 Hz, 1H, ArH), 8.34 (s, NH), 7.83 (t, J = 7.44 Hz, 2H, ArH), 7.15 (t, J = 7.12 Hz, 1H, ArH), 6.98 (d, J = 7.76 Hz, 1H, ArH), 6.87 (d, J = 8.08 Hz, 1H, ArH), 6.73 (t, J = 7.40 Hz, 1H, ArH), 4.73 (t, J = 7.40 Hz, 2H, CH_2), 4.21 (t, J = 7.80 Hz, 2H, CH_2); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 30.2, 44.2, 119.9, 121.0, 124.9, 127.2, 128.2, 128.6, 129.3, 130.9, 131.1, 131.6, 131.7, 131.9, 133.4, 134.4, 135.5, 136.5, 165.7; TOF Mass 328.2; elemental analysis: found C, 73.15; H, 4.91; N, 17.06%. $C_{20}H_{16}N_4O$ requires C, 73.17; H, 4.89; N, 17.10%.

Fluorescence quantum yield. The fluorescence quantum yield Φ_f for **1** was determined at room temperature in analytical grade CH_3CN using optically matching solutions of resublimed anthracene (Φ_f = 0.65) in ethanol as the standard at an excitation wavelength of 343 nm from a xenon lamp and the spectrofluorophotometer. The quantum yield was calculated by using eqn (1), in which Φ_{fs} is the radiative quantum yield of the sample, Φ_{fr} is the radiative quantum yield of reference, A_s and A_r are the absorbances of the sample and the reference, respectively, D_s and D_r the areas of emission for the sample and reference, Ls and L_r are the lengths of the absorption cells, and N_s and N_r are the refractive indices of the sample and reference solutions (pure solvents were assumed).

$$\Phi_{fs} = \Phi_{fr} \times \frac{1 - 10^{-A_r L_r}}{1 - 10^{-A_s L_s}} \times \frac{N_s^2}{N_r^2} \times \frac{D_s}{D_r} \qquad (1)$$

Acknowledgements

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