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ARTICLE TYPE

Water Soluble Perylene Bisimide and its Turn Off/On Fluorescence are Used to Detect of Cysteine and Homocysteine

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Perylene derivatives (P) in presence of melamine (M) have envisioned as a novel fluorescent probe for mercury ion and form selective complex (PMHg) with mercury ion at neutral medium (pH=7). This chemo-sensing ensemble probe exhibits high selectivity and sensitivity for thiol-containing amino acids, and detects cysteine and homocysteine from healthy to abnormal levels under physiologically-relevant conditions.

Among twenty amino acids, bio thiols have important role in maintaining the appropriate reducing environments of proteins, cells, organisms, and act as redox regulator.[1] Cysteine (Cys) and homocysteine (Hcy) are essential low molecular weight amino thiols that are involved in protein synthesis, detoxification, metabolism, and required for the growth of cells and tissues in living systems.[2] Increasing levels of Cys are associated with neurotoxicity,[3] and Cys deficiency causes many syndromes such as slow growth rate, hair depigmentation, oedema, skin lesions, weakness, lethargy, and liver damage.[4] Excess of Hcy results a risk factor for cardiovascular[5a] and Alzheimer's disease,[5b] neutral tube defects, inflammatory bowel disease, complications during pregnancy, and osteoporosis.[5c] Therefore, it is necessary to develop efficient methods for the detection of Cys/Hcy in physiological media for academic research as well as clinical applications.[6] By taking consideration of strong nucleophilicity of thiol group, few efforts have been addressed to develop colorimetric and fluorometric detection of Cys/Hcy.[7] Several pathways including the Michael addition,[8] cleavage reactions,[9] cyclization with aldehydes,[10] and others [11] have also been tried to serve the purpose. In spite of their sensitivities towards thiol-containing compounds, these probes suffer the key limitations: (a) irreversible due to the formation covalent bond, (b) interferences from other thiols and (c) not working in the aqueous medium.

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† Electronic Supplementary Information (ESI) available: Experimental details for the preparation of **PM12** solution. Plot of change of emission intensity, Benesi-Hildebrand analysis, quantitative estimation of Cys and Hcy in blood serum and urine sample. See DOI: 10.1039/b000000x

Fluorescent chemosensors based on "turn on" method have attracted much attention because of their high sensitivity and high potential in biological imaging. In contrast to covalent interaction,[12] chemo-sensing ensemble[13] formed through non-covalent interactions is considered to be powerful sensing platform for biothiols. Electrophilic metal cation such as Hg^{2+} or Cu^{2+} are responsible to make chemo-sensing ensemble by interacting thiol groups with the metal cations leading to the dissociation of ensemble.[14]

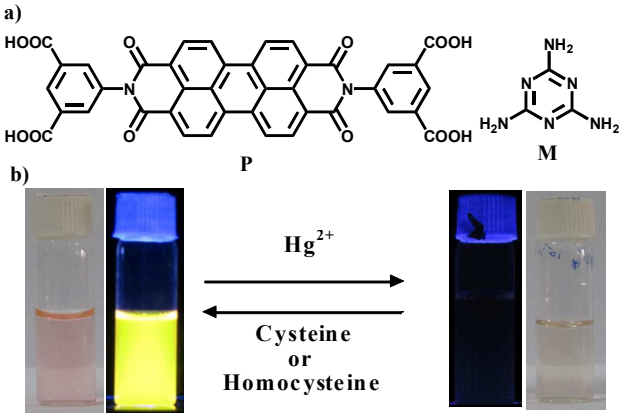

Fig. 1 (a) Chemical structure of **P** and **M**. (b) Schematic illustration for the sensing of biothiol (Cys, Hcy). In the presence of mercury ions, **P** of **PM12** complex coordinate to Hg^{2+}; upon addition of Cys/Hcy Hg^{2+} was extracted and the emission for **PM12** complex regained. Concentrations of **PM12**: 1×10^{-5} M; Hg^{2+}: 2×10^{-5} M; **PMHg**: 1×10^{-5} M; Cys: 2×10^{-5} M; Hcy: 2×10^{-5} M, in HEPES buffered (pH 7.0) water.

Perylene bisimide derivatives are important chromophores owing to their extensive use as pigments as a result of their high thermal stability and chemical inertness.[15] Because of their high quantum yield and excellent photostability, these derivatives have been considered to be the best fluorophores for single molecular spectroscopy.[16] However, most of them are not soluble in water due to the presence of large hydrophobic π conjugated ring.[17] We report here water-soluble chemo-sensing probe (**PM**), composed of perylene derivative (**P**) and melamine (**M**), which can detect Hg^{2+} via 'turn off' fluorescence.

In the presence of **M**, **P** becomes more soluble to form an aqueous mixture **PM12** (molar ratio is 1:2, 1×10^{-5} M) which

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generates very bright yellow luminescence (Fig. 1b). UV-Vis spectra of **PM12** (10 μM) in HEPES buffer exhibit bands at 452 nm, 499 nm and 536 nm and these are obvious signature of monomeric **P**. To check the optical property of **PM12**, aqueous solution of individual metal ion (Na^+, K^+, Mg^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Ag^+, Zn^{2+}, Hg^{2+}, Fe^{2+}, Ca^{2+}, Cd^{+2} and Pb^{+2} ions) has been added to the solution of **PM12** (Fig. S1).[18] Only three metal ions(Cu^{2+}, Pb^{+2} and Hg^{2+}) induce significant changes of the absorption spectra (Fig. S2), implying their responses to **PM12** under the experimental condition. To investigate the binding property of **PM12** toward the Hg^{2+} ion, titration experiments were carried out as shown in Fig. S3. On addition of Hg^{2+} ion to the solution of **PM12**, the absorption intensities at 499 nm and 536 nm are gradually diminished with the generation of new peak at around 556 nm and the intensity the peak at 452 nm is more or less unchanged. These changes are seen up to the addition of 2 equiv of Hg^{2+} ion, implying that carboxylic groups in **PM12** coordinate to Hg^{2+} ion forming a **PM12**/Hg^{2+} **(PMHg)** complex in this case. The new peak at 556 nm is a characteristic absorption band of the **PMHg** complex and can be used to diagnose whether the **PM12** binds to Hg^{2+} ion or not.

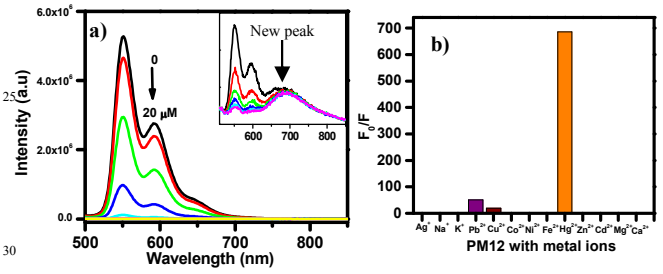

Fig. 2 (a) Fluorescence titration of **PM12** with Hg^{2+} in HEPES buffer solution at room temperature ([**P**] = 1 × 10^{-5}, [**M**] = 2 × 10^{-5} M. (b) Fluorescence spectra profiles of **PM12** (10 μM P) in HEPES buffer solution with various metal ions (20 μM). Inset spectra shows the gradual generation of new band.

Fluorescence spectra of **PM12** (λ_{exc} = 470 nm) show the characteristic emission band at 551 nm and 590 nm, indicating P is free from π–π stackings. Gradual addition of Hg^{2+} to the solution of **PM12** (10 μM), the quenching of emission bands is instantaneously observed (Fig. 2a), it completes at 2 equivalent of Hg^{2+} and the association constant of **PM12**/Hg^{2+} complex is 6.84x10^5 M^{-1}. Except three metal ions (Pb^{+2}, Cu^{2+} and Hg^{2+}), flourogenic responses (Fig. S4) of **PM12** to other metal ions do not show significant spectral change even in the presence of 100 equiv each of other metal ions. It is noteworthy to mention that **PM12** shows higher fluorescence quenching towards Hg^{2+} rather than paramagnetic Cu^{2+} and Pb^{+2} (Fig. 2b). Upon addition of Hg^{+2} ions, -COOH groups of PM12 become deprotonated, and the resulting perylene moieties get aggregated to show the quenching of monomeric emission which is compensated by a weak excimer emission observed at higher wavelength (690 nm, inset of Fig.2a).

To evaluate the selectivity of non-fluorescence ''chemo-sensing probe'' (**PMHg**) towards amino acids or peptides, the fluorescence studies in HEPES buffer solution have been performed (molar ratio of **PM12**:Hg^{2+} was 1:2 to quench the fluorescence completely). Among all amino acids, only two thiol-containing amino acids (L-Cys and L-Hcy) have surprisingly recovered the intensity of fluorescence in the solution (Fig. S6). Whereas, other amino acids even at 100-fold higher concentration of Cys and Hcy do not induce any increase in the fluorescence intensity of the solution. Significantly, other thiol containing compounds (N-acetyl-L-cysteine, glutathione, 3-mercapto-propanoic acid) do not have any interference (Fig. S7). This important result implies the practical application of the detection of Hcy and Cys quantitatively in the presence of other amino acids in biological samples, indicating its diagnostic potentiality of novel **PMHg** probe.

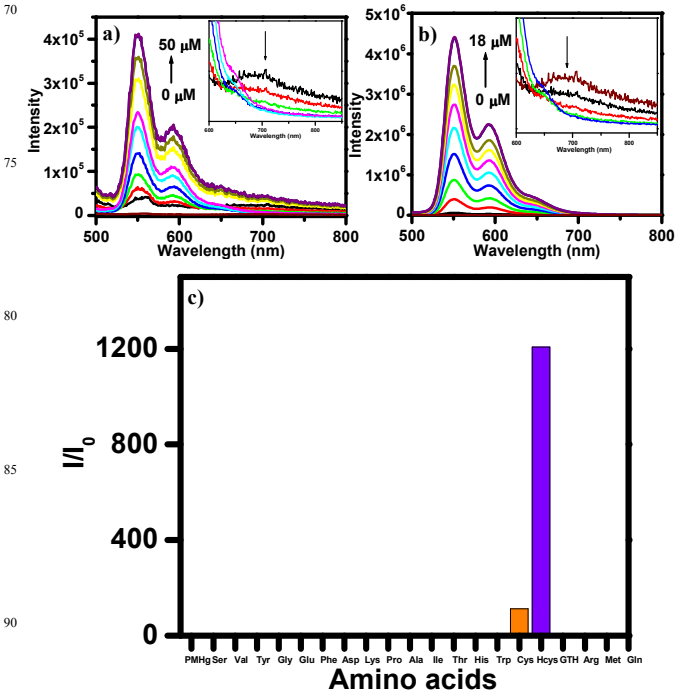

Fig. 3 Fluorescence titration of **PMHg** upon addition (a) Cys and (b) Hcy. Inset spectra show the gradual decrease of charge transfer band [λ_{ex} = 470 nm, path length = 10 mm]. (c) Fluorescence responses of **PMHg** upon addition 100 equivalent of various amino acids and peptides.

Titration spectra of **PMHg** with Cys and Hcy in HEPES buffer at pH 7 (Fig. 3a-b) have revealed that with increasing the concentration of Cys/Hcy, the fluorescence intensity at 551 nm gradually are enhanced (Fig. S8). The remarkable increase of the fluorescence is also perceived by the naked eye either in normal light or under UV lamp (Fig. 1b). The UV-Vis titration experiment also bears the response of **PMHg** with Cys or Hcy. The characteristic emission peaks of **PM12** are appearing, concomitantly, excimer emission band (690 nm, inset of Fig.3) is diminishing. It points out that there is no chemical reaction between **PM12** and Cys/Hcy. The apparent binding constant for the interaction of **PMHg** with Cys/Hcy at pH 7 is calculated from the Benesi-Hildebrand analysis (Fig. S9, 1.45x10^4 M^{-1} for Cys and 7.46x10^5 M^{-1} for Hcy). Important outcome is that the probe **PMHg** is able to detect reversibly both Cys and Hcy at both healthy and abnormal levels (Fig.S10-S14).

The switching behaviour of our sensor was further tested inside the HeLa cells with the use of a confocal fluorescence microscopy. **PM12** was not toxic to cells as observed by the MTT assay (Fig. S15). Bright-field measurement also confirmed the

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viability of cell throughout the experiments. Incubation of HeLa cells with 10 μM of **PM12** for 0.5 h at 37°C produced bright fluorescence (Fig. 4b). After the cells were subsequently supplemented with Hg^{2+} solution (20 μM) for 30 min at 37°C, complete quenching of fluorescence was noticed (Fig. 4c). Consequently, HeLa cells were further incubated with Cys for 30 min at 37°C, a remarkable increase of intracellular fluorescence was observed (Fig. 4d). The images reveal that the fluorescence signals are localized in the perinuclear/cytoplasmic space of the cells, providing a subcellular distribution of Cys. The study further indicates that **PM12** is membrane permeable, which makes this sensor valuable for cellular sensing applications. Moreover, from the bioimaging of cells (Fig. 4c), we have found that **PMHg** does not show fluorescence enhancement, suggesting that other biomolecules inside the cellular environment do not effect the fluorescence property of **PMHg** suggesting the specificity of our sensor

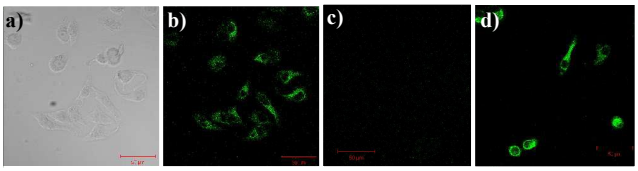

Fig. 4 Confocal fluorescence images of Hg^{2+} in Hela cells (Zeiss LSM 510 META confocal microscope, 40 × objective lens). (a) Brightfield transmission image of HeLa cells. (b) Fluorescence image of HeLa cells incubated with **PM12** (10 μM). Further incubated with addition (c) 2 equiv $HgCl_2$. (d) Again treated with Cys (2 equiv.). Scale bar on each image is 50 μm.

Encouraged by the sensing behaviour of **PMHg** in vitro and in cells, we have measured the detection capability of Cys or Hcy in human blood plasma as well as urine quantitatively.[19] Due to mal-functioning of the cellular processes, changes in the amount of low molecular mass (thiols and disulfides) in body fluids are observed. Analysis of the amount of biothiols is important. As the concentration of biothiols is very high in both serum and urine, the biological samples are diluted before the assays performed. The determined biothiol concentrations are listed in Table S1-4. By using the Cys calibration curve as the standard, the unknown endogenous amounts of thiols in diluted urine or diluted serum have been determined. The calculated thiol values for the undiluted urine and serum samples by using the Cys calibration curve show 50 μM and 245 μM respectively. These values provide the normal range of value in urine and serum. Because of the presence of much higher concentrations of Cys in urine or serum, we believe this value determined using Cys calibration curve should be accurate. Recovery of added known amounts of Cys to the urine or serum samples is from 96 % to 101 %. These results suggest the potentiality of this probe for thiol determination in practical applications. To further prove the better quality of our system, we have compared the fluorescence intensity between the Cys calibration curve and the urine or serum samples (with original plus spiked Cys), as shown in Fig. S12a-b. The total amount of Hcy content in the commercial blood by similar way as for cysteine is found to be 15 μM (Fig. S14a-b). It is expected that the sensor can be used in thiol detection for biological samples.

In conclusion, we have successfully developed a supramolecular as well as chemo-sensing probe (**PMHg**) for the detection of Cys/Hcy over glutathione at physiological pH. This approach includes the coordination of Hg^{2+} with Cys/Hcy. The fluorescence technique is selectively used to measure Cys or Hcy in the presence of other biothiols in human blood plasma and urine.

Experimental

General:

Reagents were purchased from sigma-aldrich and used as received and **P** was synthesized according to our earlier reported procedure.[17] For all aqueous mixtures and for spectroscopic studies, water of HPLC grade was used and for the preparation of solution for sensing purpose HEPES buffer (pH = 7) were used. ^{1}H-NMR spectra were recorded at room temperature on 300 MHz spectrometers (Bruker). Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry was carried out with Bruker Daltonics FLEX-PC using CHCA as a matrix. The UV-Vis spectra of all samples were studied with Hewlett-Packard UV-Vis spectrophotometer (model 8453). Fluorescence studies for all samples, prepared in a sealed cuvette, were carried out with Horiba Jobin Yvon Fluoromax 3 instrument at excited wavelength 470 nm and spectra were recorded with slits 2/2 nm.

*Synthesis of N, N'-Di-(phenyl-3, 5-dicarboxylic acid)-perylene-3,4:9,10-tetracarboxylic diimide(**P**)*:[17]

2.00g (5.10 mmol) of perylene tetracarboxylic dianhydride, 2.308g (12.74 mmol) 5-aminoisophthalic acid and 10g of imidazole were heated at 130°C for 6 hrs under Ar atmosphere. Then 150 mL of ethanol was poured into the hot mixture and refluxed for overnight. Then the reaction mixture was cooled to room temperature precipitate out. The precipitate was filtered and washed with ethanol (50 mL, 3 times). The product was dried at 70°C under vacuum oven to get deep red powder like pdt. Yield: 80%. ^{1}H NMR (300 MH_z , D_2O) δ ppm: 8.52 (s, 2H) , 8.14 (s, 4H), 7.23 (m, 8H); IR (KBr) ν_{max} = 746, 808, 851, 1115, 1254, 1355, 1575, 1662, 1696, 2927, 3152 cm^{-1}; MALDI-TOF-MS m/z calculated for $C_{40}H_{18}N_2O_{12}$: 718.58, found: 741.14 $[M+Na^+]$. UV/Vis (H_2O): λ_{max}/nm ($\varepsilon/M^{-1}cm^{-1}$) 532 (27653), 499 (19416) , 464 (8476); Fluorescence (H_2O): λ_{max}/nm: 551, 594 and Fluorescence quantum yield, Φ_f = 0.21.

Preparation of stock solution**:**

PM12 solution was prepared in HEPES buffered solution by mixing melamine solution (2 x 10^{-5} M) to the solution of **P** (1 x 10^{-5} M) in HEPES buffer. The solution was kept at 15°C for 12 hrs to complete the formation of supramolecular polymer. Then the solution was used for the synthesis of the chemosensing ensemble probe selectively with Hg^{2+} among all other metal ions. Stock solutions of Hg^{2+}, GSH, Cys, Hcy and various other amino acids (1 mM) were prepared by dissolving in water.

For sensing the thiol in urine sample, the urine sample collected from a healthy adult volunteer was used as the urine stock solution. The test solution was prepared by adding the requisite amounts of stock solutions together, diluting with pH 7.0 HEPES buffer. The solution was stirred for 30 seconds and fluorescence spectra were recorded. The final urine concentration in the test solution is 10-fold diluted.[20] All experiments related to blood serum and urine were performed in compliance with the

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institutional guidelines approved by the Institute Bio-Safety (IBS) committee of IACS, Kolkata.

For sensing the thiol in blood serum sample, fetal bovine serum was used. In serum, most of the thiol-containing amino acids were generally bound to proteins or other thiols and were in the disulfide form.[21] Those low-molecule mass thiols had been become free in the serum after reduction with suitable reagents and resulting solution were used for analysis. In the present study, the disulfide bonds were reduced in order to release the protein-bound thiols by addition of triphenylphosphine as catalyst according to literature report.[22] The final serum concentration in the test solution was 20-fold diluted. For recovery studies, GSH having known concentrations were added to the samples and the total thiol concentrations were then determined.

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