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A red fluorescent turn-on probe for hydrogen sulfide and its application in living cells†

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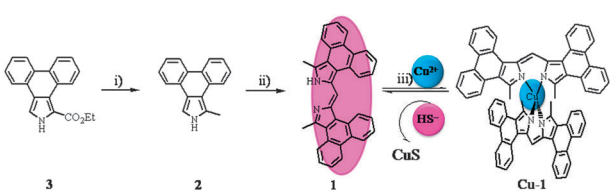
A novel selective fluorescent chemosensor 1 has been synthesized with a phenanthrene-fused dipyrromethene structure. Selective binding of Cu^{2+} by 1 results in a complex that displays high selectivity and sensitivity for H_2S. The signal transduction occurs *via* reversible formation–separation of the Cu-1 complex and CuS. Its potential utility for biological applications was confirmed by fluorescence imaging of H_2S in live cells.

Recently, there has been a strong focus on developing artificial optical receptors for hydrogen sulfide, a toxic gas which provides the characteristic smell of rotten eggs.[1] Studies have identified H_2S as the third endogenous gaseous transmitter, in addition to nitric oxide (NO) and carbon monoxide (CO).[2] H_2S or aqueous sulphide elicits diverse physiological responses, including the modulation of blood pressure and the reduction of ischemia reperfusion injury, the exertion of anti-inflammatory effects, the reduction of the metabolic rate, and is the cause of many diseases, possibly including Alzheimer's disease.[3] Therefore, it is important to develop a rapid and sensitive method for monitoring hydrogen sulfide in aqueous media and biological systems. A large number of techniques for recognizing H_2S have been developed, including colorimetric and electrochemical assays, gas chromatography, and metal-induced sulfide precipitation.[4] The utilization of known metal-anion affinities is an important method for sensing anions.[5] CuS is a very stable compound with a low-solubility product constant and has considerable potential in this regard. There are very few examples of reversible sensors exploiting this metal-anion affinity, however.[6] Dipyrromethene analogues can form extremely stable complexes with boric acid derivatives yielding BODIPY dyes. In recent years, these compounds have been investigated as fluorescent probes for biological imaging, as emitter materials in organic light emitting diodes, and as absorber materials for organic solar cells.[7] The photophysical properties of the transition metal complexes of these ligands have only rarely been studied.[8] Recently, we have studied the use of fluorescence imaging for the nondestructive detection of specific biomolecules in live cells or tissues using readily available instrumentation.[9] The following characteristics are highly desirable for intracellular imaging: (i) selectivity and sensitivity toward a specific ion; (ii) fluorescence maxima in the NIR region (650–900 nm).[10] The Nagano group have reported a copper-based coordination complex in which a Cu^{2+} center can be released by binding with H_2S to induce fluorescence from the π-system of the ligand.[11] Unfortunately, the absorption and emission bands of these probes lie at *ca.* 500 nm outside the optical penetration window in biological tissue. Very few examples of fluorescent probes for H_2S intracellular imaging at the red end of the visible region or in the NIR region have been reported.[12] Herein, we report the use of a phenanthrene-fused dipyrromethene to form a novel fluorescent sensor **1**. The ligand exhibits excellent optical properties and can be used as a fluorescence turn-off chemosensor for Cu^{2+} and as a turn-on probe for Zn^{2+}. **Cu-1** displays high sensitivity and selectivity for H_2S in the presence of other possible competitive anions due to the formation of CuS through metal-induced sulfide precipitation.

The synthesis of **Cu-1** is outlined in Scheme 1. Ethylphenanthro[9,10-*c*]-pyrrole-1-carboxylate **3** was synthesized in 61% yield according to a published procedure.[13] 1-Methylphenanthro[9,10-*c*]-pyrrole **2** was prepared through the treatment of **3** with lithium aluminium hydride in THF solution. The trifluoroacetic acid catalyzed condensation reaction of **2** with triethyl orthoformate gave **1** in 26% yield.[14] **Cu-1** can be obtained quantitatively by treatment of **1** with copper(II) acetate in THF solution.

The UV/visible absorption and emission spectra of **1** and **Cu-1** were measured in CH_2Cl_2. The absorption spectrum of **1** (Fig. S13, ESI†) contains a broad envelope of intensity stretching from 430–620 nm with the maximum peak centered at 550 nm. This can be



Scheme 1 Synthetic procedures for compound **Cu-1**: (i) lithium aluminium hydride in THF, reflux; (ii) trifluoroacetic acid and triethyl orthoformate in CH_2Cl_2, rt, 26%; (iii) copper(II) acetate in THF, rt, quantitative.

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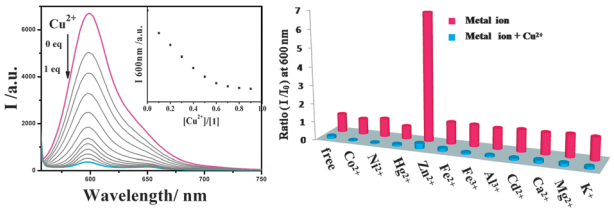

Fig. 1 (left) Changes in the fluorescence spectrum of **1** (20 μM in DMSO–H_2O, 4 : 1 v/v) as the Cu^{2+} concentration is increased. The inset shows that the emission intensity at 600 nm changes as a function of Cu^{2+} concentration. (right) The fluorescence intensity of **1** observed at 600 nm upon addition of various metal ions.

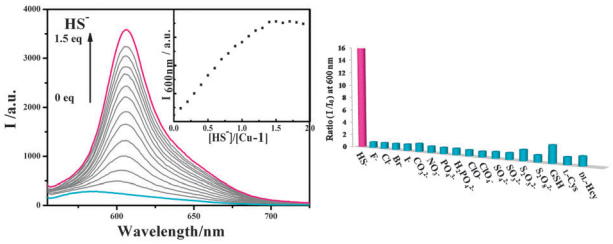

Fig. 2 (left) Changes in the fluorescence spectrum of **Cu-1** (20 μM in DMSO–H_2O, 4 : 1, v/v) as the HS^- concentration is increased. The inset shows that the emission intensity at 600 nm changes as a function of the HS^- concentration. (right) The fluorescence intensity of **Cu-1** is observed at 600 nm upon addition of various anions.

assigned to the lowest-lying π–π* transition of **1**. After binding with Cu^{2+}, the main absorption intensity of **Cu-1** is red shifted with three well-defined peaks at 544, 563 and 593 nm. TD-DFT calculations predict that these transitions are mainly associated with the ligand to metal charge transfer transitions associated with the metal center (Fig. S12 and 13, ESI†). The fluorescence spectrum of **1** in DMSO–H_2O (4 : 1, v/v) contains an intense band at 600 nm (λ_{ex} = 540 nm; Φ_1 = 0.008). The fluorescence intensity of **1** is quenched upon the addition of increasing concentrations of Cu^{2+} (Fig. 1). The addition of a series of different ions, such as 10 eq. of Ni^{2+}, Zn^{2+}, Fe^{2+}, Fe^{3+}, Al^{3+}, Cd^{2+}, and 100 eq. of Ca^{2+}, Mg^{2+}, K^+, did not generate similar quenching, although small influences were observed upon the addition of 1 eq. of Co^{2+} (I/I_0 = 0.86) and 1 eq. of Hg^{2+} (I/I_0 = 0.71) (Fig. 1). To evaluate the utility of **1** as a Cu^{2+}-selective fluorescent sensor, ion interference experiments were carried out. As shown in Fig. 1, there is no interference in the Cu^{2+} response in the presence of an appropriate series of metal ions. There is an immediate fluorescence quenching response upon addition of 1 eq. of Cu^{2+} in the presence of most other biologically relevant ions, such as Ni^{2+}, Fe^{2+}, Ca^{2+}, Mg^{2+}, K^+. However, there is a slower response time of about 5 h after the addition of Cu^{2+} in the presence of Zn^{2+}, Fe^{3+} and Al^{3+} ions. This suggests that **1** reacts with the Cu^{2+} ion with high selectivity in aqueous solution, but the ion interference experiments demonstrate that the reaction can be slow for kinetic reasons, with the interaction with Zn^{2+} being the most relevant in a biological context.

Upon interaction with Cu^{2+} (0.5 eq.), the system exhibits significant fluorescence quenching (75% quenching, $\Phi_{1\text{–Cu}}$ = 0.002) (Fig. 1). The most obvious explanation for this is the coordination of the d^9 Cu^{2+} center, since the S1 state is likely to arise from either the d → d or ligand to metal charge transfer transitions associated with the metal center rather than the lowest energy π → π* transition of the ligand that is responsible for the dye properties of the BODIPY fluorophore.[15] A 1 : 2 complexation stoichiometry was confirmed using a Job plot (Fig. S7, ESI†). In addition, when different concentrations of Zn^{2+} ions are added in DMSO–H_2O solution (4 : 1, v/v), the fluorescence intensity of **1** gradually increases by approximately 7-fold at 600 nm ($\Phi_{1\text{–Zn}}$ = 0.1) (Fig. S8, ESI†). Chelation of the d^{10} Zn^{2+} results in a decrease in the conformational flexibility of ligand **1** and hence in the rate of non-radiative decay.[16] No interference is observed in the presence of 1 eq. of Co^{2+}, Hg^{2+}, 10 eq. of Ni^{2+}, Zn^{2+}, Fe^{2+}, Fe^{3+}, Al^{3+}, Cd^{2+}, and 100 eq. of Ca^{2+}, Mg^{2+}, K^+, respectively (Fig. 1). This demonstrates the utility of **1** as an ion-selective fluorescence turn-on sensor for Zn^{2+}.

As expected, **Cu-1** in DMSO–H_2O (4/1, v/v) solution has very weak fluorescence with a quantum yield of 0.002. Upon addition of 1.5 eq. of HS^-, the fluorescence intensity increases dramatically. A fluorescence enhancement factor of approximately 14-fold is observed at 600 nm (Fig. 2). Both the intensity and shape of the emission band closely match those of **1** and, therefore, can be attributed to the release of ligand **1** upon the formation of a more stable CuS species with a low-solubility product constant $\kappa_{sp} = 6.3 \times 10^{-36}$. Almost no change is observed in the fluorescence intensity of **Cu-1** after the addition of 100 eq. of common inorganic anions, such as F^-, Cl^-, Br^-, I^-, CO_3^{2-}, NO_3^-, PO_4^{3-}, $H_2PO_4^-$, ClO^-, ClO_4^-, and 100 eq. of various sulfur oxyanions, such as SO_3^{2-}, SO_4^{2-}, $S_2O_3^{2-}$, $S_2O_8^{2-}$, and 10 eq. of other biothiols, including reduced glutathione (GSH), L-cysteine (L-Cys), and DL-homocysteine (DL-Hcy) (Fig. 2 and Fig. S9, ESI†). The fluorescence spectra were recorded within 5 min of the addition of the HS^- anion, and subsequently remained almost unchanged for the next 15 min. H_2S could be detected down to 1 μM, when **Cu-1** was employed at 0.2 μM in a DMSO–H_2O (4 : 1, v/v) solution. Since the average endogenous H_2S level is in the μM range, this demonstrates that the **Cu-1** can act as a stable and rapid real-time chemosensor for H_2S in live cells (Fig. S10, ESI†), with high selectivity and sensitivity.

B3LYP geometry optimizations were carried out with 6-31G(d) basis sets to determine the configurations of **1** and **Cu-1**. As anticipated, the Cu^{2+} ion of **Cu-1** is predicted to bind tetrahedrally to two phenanthrene-fused dipyrromethenes through four nitrogen atoms with Cu–N bond lengths of 1.97 Å (Fig. 3). The π-system of **1** is not planar. A dihedral angle of 15.46° is predicted between the two phenanthrene pyrrole planes due to steric effects. A larger dihedral angle of 20.1° is predicted for **Cu-1**. The key advantage of using a phenanthrene-fused dipyrromethene is that the main absorption and fluorescence bands undergo a significant red-shift into the optical window in biological tissue at the red end of the visible region, due to significant narrowing of the HOMO–LUMO gap (Fig. S11, ESI†). TD-DFT calculations for **Cu-1** predict that bands

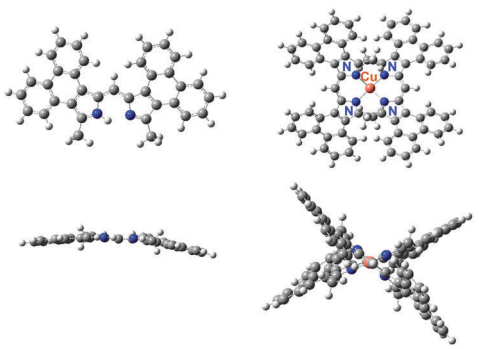

Fig. 3 The calculated energy-minimized structures of **1** (left) and **Cu-1** (right).

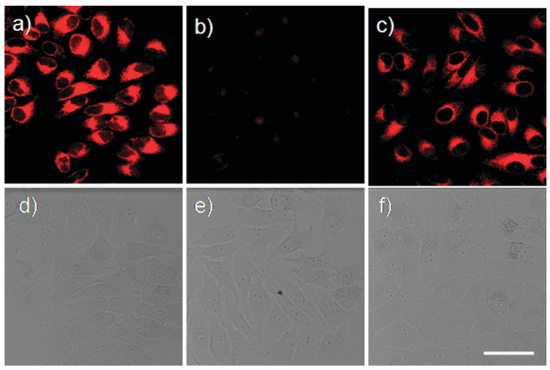

Fig. 4 Confocal fluorescence images of living HeLa cells: (a) cells loaded with 10 μM **1** for 30 min. (b) Cells loaded with 50 μM **Cu-1** for 30 min. (c) **Cu-1**-loaded cells incubated with 50 μM NaHS for 30 min. (d–f) Brightfield images of living Hela cells in panels (a)–(c) confirm that the cells are viable throughout the imaging experiments. Scale bar = 50 μm.

associated primarily with the one-electron transitions between MOs derived from the HOMO and LUMO of **1** are forbidden after the metal complex is formed (Fig. S12, ESI†).

The potential utility of **Cu-1** as a probe for HS^- within living cells was evaluated (Fig. 4).[11] After incubating HeLa cells with 10 μM of **1** for 30 min at 37 °C, intense intracellular red fluorescence was observed upon excitation at 543 nm as in the microscope image (Fig. 4a). When 50 μM of **Cu-1** was added to the growth medium for 30 min at 37 °C, no fluorescence emission was observed from the intracellular region (Fig. 4b). In contrast, when 50 μM of HS^- was added for 30 min at 37 °C, intense intracellular fluorescence was observed as in the microscope image (Fig. 4c). Bright field measurements with both HS^- and **Cu-1** after treatment confirmed that the cells are viable throughout the imaging experiments (Fig. 4d–f). These results demonstrate that **Cu-1** is an effective agent for imaging intracellular HS^-.

In addition, large signal ratios ($I_2/I_3 > 20$) are observed between the cytoplasm (region 2 in Fig. S14, ESI†) and the nucleus (region 3) due to weak nuclear uptake of compound **1**. This leads to exclusive staining in the cytoplasm (Fig. S14, ESI†). Three-dimensional fluorescence images of HeLa cells stained with compound **1** have also been obtained (Fig. S15, ESI†). These results indicate that **1** is cell-permeable and **Cu-1** can respond to variations in the concentration of intracellular HS^- anions.

The cell viability of **1** and **Cu-1** was evaluated through an MTT assay (Fig. S16, ESI†). Cell viability was monitored for 24 h after treatment with the two compounds over a wide range of concentrations (5–100 μM). This demonstrates that **1** and **Cu-1** do not negatively affect the cell viability over the range of concentrations studied, and that there is no evidence of cytotoxicity. **Cu-1** can, therefore, clearly be used for the intracellular detection of the HS^- anion.

In summary, a phenanthrene-fused dipyrromethene analogue has been found to act as a red fluorescent turn-on sensor for the HS^- anion by utilizing the displacement method. The chemosensor exhibits high selectivity for HS^- in the presence of other common anions including various sulfur oxyanions. After interaction with HS^-, the red fluorescence of **1** is again observed. An inorganic reaction results in a dosimetric anion determination based on a formation–separation process of the **Cu-1** complex and CuS formation. In addition, **Cu-1** can also be used as a fluorescence turn-on chemosensor for HS^- in HeLa cells, thus demonstrating its utility for studying the effect of HS^- in biological systems. These results pave the way to a new generation of molecular recognition systems in the optical penetration window for biological tissues, since a very wide range of fused-ring-expanded dipyrromethenes can be formed.

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