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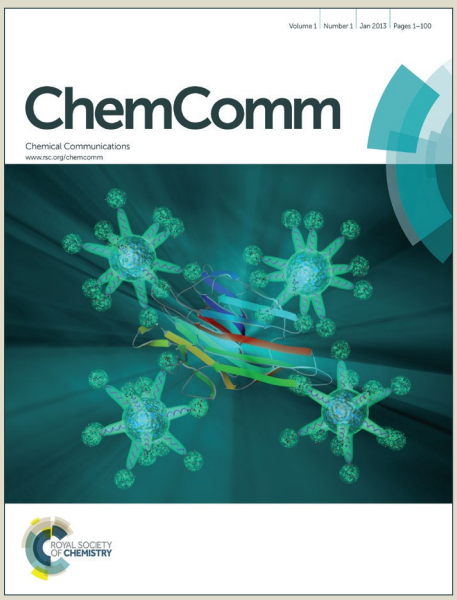

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COMMUNICATION

A Fluorescent Probe for Relay Recognition of Homocysteine and Group IIIA Ions Including Ga(III)

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

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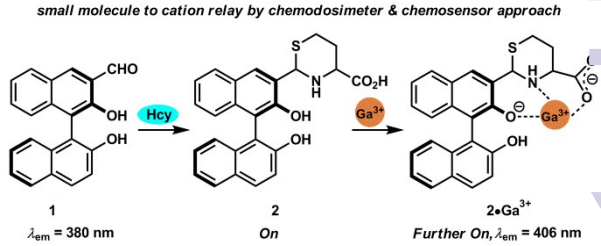
Challenging relay recognition of Hcy and Ga^{3+}, has been realized for the first time. Chemodosimeter 1 bearing 1,1'-binaphthyl skeleton is a fluorescence turn-on probe for Hcy over Cys. The system (2) generated from the recognition of Hcy exhibited futher fluorescence enhancement for Ga^{3+}, which is the first example for specific fluorescent recognition of this metal ion.

Cysteine (Cys), homocysteine (Hcy), and glutathione (GSH) exist broadly in living organism, and play essential roles in many biological processes.[1] Abnormal levels of these biothiols can also result in some diseases. Specifically, elevated levels of Hcy in plasma is a risk factor for folate and cobalamin deficiencies, Alzheimer's and cardiovascular diseases.[2] Early diagnosis through versatile detection of these biothiols, is thus very important. Pleasingly, the development of fluorescence methods has received considerable attention due to their sensitivity and easy-to handling. Small-molecule fluorescent probes by molecular recognition or thiol specific reactions, have appeared.[3] Moreover, fluorescent probes for specific or discriminative recognition of GSH and Cys have been extensively studied.[4] However, most of these probes have low selectivity between Cys and Hcy due to their highly similar structures.[5] Only a few probes have shown specificity for Hcy.[6] The discrimination of Hcy over Cys is still a significant challenge.

Gallium is a soft, silvery metal with modest conductivity[7] and extensively used in semiconductor industry, fuel storage and chemical synthesis.[8] Since Ga is the element following Al in Group IIIA, its chemical behavior is similar to that of Al, and it always presents as a contaminant of Al compounds.[9] Moreover, Ga^{3+} ion has shown high affinity for tumors: its nitrate salt has been used as an antitumor pharmaceutical.[10] So the development of sensing methods for gallium ions has created the need and academic interests.[11] To our knowledge, to date, there is no report about highly selective fluorescence probe for Ga^{3+}, which therefore still remains a unmet task.



Scheme 1 Relay fluorescence recognition of Hcy and Ga^{3+}.

Bifunctional probes, which utilize a single probe to detecting two analytes by distinct fluorescence responses, have become a research focus.[12] In connection with continuing research on this kind of probes,[13] we proposed relay recognition strategy as a new pathway.[14] Since the sequence-specific recognition of various species in biological systems is very important,[15] these relay probes have become an tool for studying related physiological and pathological processes. In this work, relay recognition of Hcy and Ga^{3+} with the novel off–on–on fluorescence mode (Scheme 1), was first disclosed based on the sequential chemodosimeter and chemosensor approaches.

Cyclization reaction with the aldehyde group in probes has been used to sensing Cys/Hcy over GSH.[16] We thus designed a simple chemodosimeter (**1**) based on the unique 1,1'-binaphthyl fluorophore[17] tethered to an aldehyde moiety. The probe was easily synthesized from 1,1'-binaphthol (Scheme S1, ESI†), and characterized by ^1H, ^{13}C NMR and MS (Figs. S5–S8). As expectedly, a six-membered thiazinane **2** would be obtained by the reaction of **1** with Hcy. According to our previous research,[13b,18] α-amino acid moiety in **2** may response to Group IIIA cations including Ga^{3+}. In this regards, a new relay combination from small molecule to cation could be realized.

As shown in Fig. 1, **1** shows a monomer emission band at about 380 nm of the 1,1'-binaphthyl fluorophore when excited at 330 nm in ethanol–HEPES (v/v = 98:2, pH = 7.0) solution.[19] Next, Thr, Asn, Glu, Asp, Tyr, Lys, Arg, His, Gln, Gly, Ala, Leu, Ile, Val, Pro, Phe, Met, Trp, Ser, GSH, Cys, and Hcy were used to investigate the selectivity of **1** by fluorescence spectra (Fig. 1a). Compared to other amino acids examined, the fluorescence

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Electronic Supplementary Information (ESI) available: Spectral data, copies of $^1H/^{13}C$ NMR, ESI-MS, ESI-TOF MS, and HRMS. See DOI: 10.1039/x0xx00000x

intensity clearly increased only in the presence of Hcy. To our delight, further fluorescence enhancement did happen upon the addition of Ga^{3+} to the solution of **2**, which was in situ generated after the recognition of Hcy (Fig. 1b). Moreover, Li^{+}, Na^{+}, K^{+}, Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}, Pb^{2+}, Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Hg^{2+}, Fe^{3+}, Al^{3+}, and In^{3+} were used to measure the selectivity. Compared to other cations examined, only Group IIIA cations significantly increased the emission with a bathochromic shift. These results showed that probe **1** displayed high selectivity toward Hcy, and the resulting relay system from **1** + Hcy can sensing Al^{3+}, Ga^{3+}, and In^{3+} in ethanol–HEPES (v/v = 98:2, pH = 7.0) solution. The designed relay recognition with an off–on–on fluorescence change has been achieved.

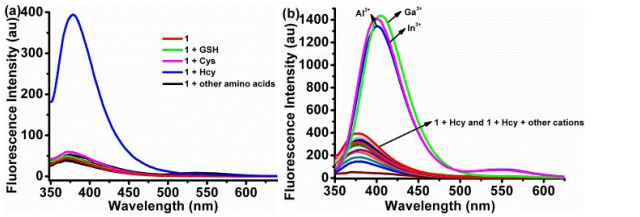

Fig. 1 (a) Fluorescence responses of **1** (20.0 μM) with various amino acids (550.0 μM) in ethanol–HEPES (v/v = 98:2, pH = 7.0) solution (λ_{ex} = 330 nm); (b) fluorescence spectral changes of **1** + Hcy (20.0 μM + 550.0 μM) with various metal ions (100.0 μM) in ethanol–HEPES (v/v = 98:2, pH = 7.0) solution (λ_{ex} = 330 nm).

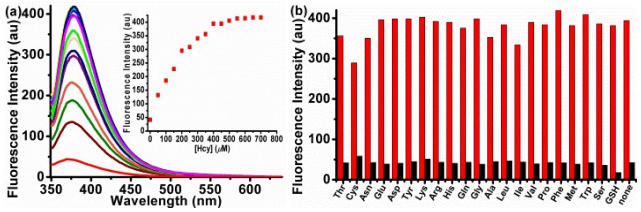

Fig. 2 (a) Fluorescence spectra of **1** (20.0 μM) upon the addition of Hcy (0–700.0 μM) (λ_{ex} = 330 nm). Inset: Fluorescence intensity at 380 nm as a function of Hcy concentration; (b) the selectivity of **1** (20.0 μM) for Hcy (λ_{em} = 380 nm). The black bars represent the emission intensity of **1** in the presence of other amino acids (550.0 μM). The red bars represent the emission intensity that occurs upon the subsequent addition of Hcy (550.0 μM) to the above solution.

The fluorescence titrations of Hcy were then conducted using a 20.0 μM solution of probe **1** in ethanol–HEPES (v/v = 98:2, pH = 7.0) (Fig. 2a). Upon the addition of Hcy to this solution, a significant increase of the fluorescence emission band centered at 380 nm was observed when excited at 330 nm. The total fluorescence enhancement factor at 380 nm was determined as 10-fold. The fluorescence quantum yield (Φ)[20] of **1** at 380 nm also increased from 1.6% to 4.8% in the presence of Hcy in ethanol–HEPES solution. The corresponding detection limit[21] was found to be 5.4×10^{-5} M for Hcy (Fig. S21). The kinetic studies of the response of Hcy (550.0 μM) to probe **1** (20.0 μM) in ethanol–HEPES solution at 37 °C were measured (Fig. S22). The observed rate constant (k_{obs}) was estimated to be 5.55×10^{-4} s^{-1} for Hcy by fitting the initial fluorescent intensity changes according to a pseudo-first-order kinetics equation. To validate the selectivity of **1** in practice, competition experiments were also measured by the addition of Hcy to the ethanol–HEPES (v/v = 98:2, pH = 7.0) solutions of **1** in the presence of other amino acids including Cys and GSH (Fig. 2b). Pleasingly, they had no obvious interference with the detection of Hcy. These results suggested that **1** can function as a specific fluorescent probe for Hcy *via* a "turn-on" response in ethanol–HEPES (v/v = 98:2, pH = 7.0) solution.

To verify the interaction of Hcy with probe **1**, ^{1}H NMR spectroscopic analysis was also investigated (Fig. S23). With the addition of Hcy to probe **1**, the signal of –CH_aO (10.22 ppm) disappeared gradually, while a methine proton was observed at 5.70 ppm. And the signal of H_b (8.60 ppm) in naphthalene ring was shifted upfield to ca. 8.20 ppm. These results implied the cyclization of Hcy with aldehyde group in probe **1** occurred. A preparation experiment was then carried out (Scheme S1), and a product was isolated indeed and further identified (Figs S9–S12) as six-membered thiazinane **2**, which is responsible for the observed fluorescence change. The corresponding synthesis with Cys was next conducted, and the expected thiazolidine **3** was also obtained and characterized analogously (Figs S13–15). Surprisingly, its fluorescence is weak in ethanol–HEPES (v/v = 98:2, pH = 7.0) solution (Fig. 1a), which is the origin of the above discrimination recognition of Hcy over Cys.

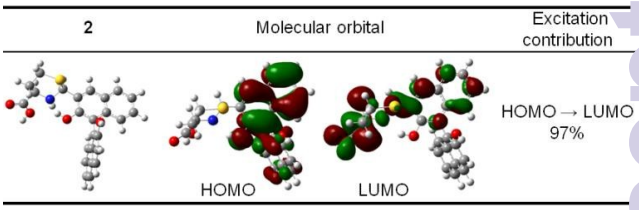

Fig. 3 The molecular orbital plots and excitation contributions of **2**.

To further understand the distinct fluorescence properties of **2** and **3**, we carried out density function theory (DFT) and time-dependent density function theory (TD-DFT) calculations with B3LYP/6-31G+ basis set using the Gaussian 09 program.[22] The optimized structures, and the molecular orbital plots of **2** and **3** are shown in Figs 3, S24–S26. The main contribution transition of **2** for the $S_0 \rightarrow S_1$ energy state comes from HOMO→LUMO (ca. 97%), which is responsible for its strong fluorescence. As for **3**, six contribution transitions to its excited state were found (Fig. S24). Among them, HOMO–1→LUMO+1 (ca. 27%) transition involves intramolecular charge transfer (ICT) process and HOMO→LUMO+1 (ca. 41%) transition involves photo-induced electron transfer (PET) process,[23] both of which may cause the fluorescence quenching of **3**. Notably, the fluorescence quenching caused by similar ICT and PET process had been observed by Yoon and co-workers recently.[6a] Altogether, the above calculation results are in good agreement with much higher fluorescence intensity of **1** + Hcy (i.e., **2**) than that of **1** + Cys (i.e., **3**).

Fluorescence titrations of Group IIIA cations were then conducted using the obtained relay probe **2** in ethanol–HEPES (v/v = 98:2, pH = 7.0) solution. Upon the addition of Al^{3+}, Ga^{3+} or In^{3+} to **2**, a significant increase of the monomer emission

band of the 1,1'-binaphthyl fluorophore[17] at 380 nm with a bathochromic shift to 400 nm (Al^{3+} and In^{3+}) or to 406 nm (Ga^{3+}) were observed (Figs S27–S29) when excited at 330 nm. The total fluorescence enhancement factors were determined as 5.8-fold for Al^{3+}, 6.8-fold for Ga^{3+}, and 5.7-fold for In^{3+}, respectively. Meanwhile, an excimer emission band at 549 nm was also observed but only in the case of Al^{3+} or In^{3+}. Thus, the differential recognition of Ga^{3+} from Al^{3+} and In^{3+} could be achieved under this condition, depending on whether the fluorescence enhancement at the excimer emission band or the enhancement of the monomer emission band at 406 nm or 400 nm would appear (Table S1). Fluorescence quantum yields (Φ) of **2** at λ_{em} = 400 nm increased from 2.2% to 15.0%, 16.6% or 16.4% in the presence of 1.0 equiv of Al^{3+}, Ga^{3+}, or In^{3+}, respectively. The corresponding detection limits were calculated to be 0.94 μM (Al^{3+}), 2.37 μM (Ga^{3+}) and 0.55 μM (In^{3+}), respectively (Figs S30–S32). To validate the selectivity of **2**, competition experiments were also conducted (Figs S33–S35). Metal ions such as Li^{+}, Na^{+}, K^{+}, Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}, Pb^{2+}, Mn^{2+}, Fe^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Hg^{2+}, and Fe^{3+} had no obvious interference, which suggested that **2** is useful for sensing Group IIIA cations, and its enhancement can be attributed to the chelation-enhanced fluorescence.[24]

Subsequently, a 1:1 stoichiometry complexation between **2** and Al^{3+}, Ga^{3+}, or In^{3+} was determined by using the Job's plot (Figs S36–S38) and Benesi–Hildebrand plot[25] (Figs S39–S41). The association constants *K* of the corresponding complexes were then calculated to be 4.72×10^{3} M^{-1} for Al^{3+}, 3.82×10^{4} M^{-1} for Ga^{3+}, and 8.2×10^{3} M^{-1} for In^{3+} by using the emission changes, respectively. Moreover, the ESI mass spectra provide additional evidence for the formation of 1:1 complex between **2** and Al^{3+}, Ga^{3+}, or In^{3+} (Figs S42–S44). To further investigation of the binding sites of **2** with Al^{3+}, Ga^{3+}, or In^{3+}, the ^{1}H NMR-titration experiments were also carried out (Figs S45–S47).

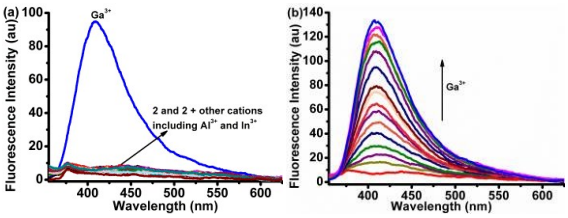

Fig. 4 (a) Fluorescence spectral changes of **2** (20.0 μM) with various metal ions (20.0 μM) in HEPES (pH = 7.0) solution (λ_{ex} = 330 nm); (b) fluorescence spectra of **2** (20.0 μM) upon the addition of Ga^{3+} in HEPES (pH = 7.0) solution. [Ga^{3+}] = 0, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 12.0, 14.0, 16.0, 18.0, 20.0 μM.

Considering the potential application, the fluorescence properties of the compound **2** and **2** + metal ions in H_2O (0.1% DMSO, v/v) solution were then studied in detail. Compared to that in ethanol–HEPES (v/v = 98:2, pH = 7.0) solution, the fluorescence intensity of thiazinane **2** quenched severely. But interestingly, compared to other metal ions examined, only Ga^{3+} caused a significant fluorescence enhancement of **2** at 409 nm (Fig. 4a). The high selectivity of Ga^{3+} over Al^{3+} and In^{3+} was probably caused by pH effect in water due to the strong hydration ability of these Group IIIA ions. Thus, the pH effect on the fluorescence intensity of **2** was investigated in detail (Fig. S48). Compared to the fluorescence intensity of **2**, the addition of Ga^{3+} within the scope of pH 4.0 and 10.5 increased the fluorescence intensity significantly. But obvious increase induced by Al^{3+} or In^{3+} was not observed. Consequently, the pH effect along with a stronger coordination of **2** with Ga^{3+} could be main reasons for specific recognition of this metal ion in HEPES solution. In subsequent experiments, a pH 7.0 solution was used as an ideal media. The addition of Ga^{3+} (1.0 equiv) to the HEPES buffer (pH = 7.0) solution of **2** resulted a 13.5-fold fluorescence enhancement at 409 nm when excited at 330 nm (Fig. 4b). In contrast, all competitive metal ions had no obvious interference with the detection of Ga^{3+} ion (Fig. S49). The corresponding detection limit was calculated to be 1.54 μM (Fig. S50). And the fluorescent quantum yield (Φ) increased from 0.03 % to 1.6 % in the presence of Ga^{3+} (1.0 equiv) in HEPES buffer solution. These results clearly indicated that **2** can be served as a turn-on probe to detect Ga^{3+} ion in water.

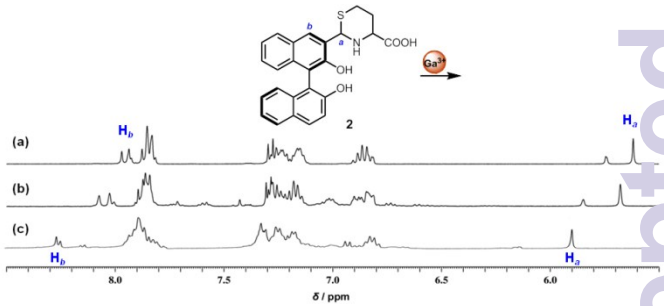

Fig. 5 Partial ^{1}H NMR (400 MHz) spectral change of the probe **2** (10.0 mM) in DMSO-d_6/D_2O (4 : 1, v/v): (a) **2** only; (b) **2** + Ga^{3+} (0.5 equiv); (c) **2** + Ga^{3+} (1.0 equiv).

Job's plot (Fig. S51), Benesi–Hildbrand plot (Fig. S52), and the ESI-TOF MS (Fig. S53) also indicated 1:1 binding mode between **2** and Ga^{3+} in HEPES (pH 7.0) solution. The association constant *K* of the complex was then calculated to be 2.29×10^{4} M^{-1} by using the emission changes at 409 nm (Fig. S52). A peak at *m/z* 861.2497 assigned to [**2** + Ga^{3+} + $3ClO_4^{-}$ + H_2O + EtOH − H^{+}]$^{-}$ was observed in negative-ion ESI-TOF MS (Fig. S53). Moreover, the similar ^{1}H NMR-titration experiments (Fig. S54) indicated the binding sites of **2** with Ga^{3+} may be –OH, –COO– and –NH–, since signals of H_a and H_b of **2** were shifted downfield upon the addition of Ga^{3+}. These results implied that **2** coordinated well with Ga^{3+} in HEPES solution as well.

We have synthesized a probe **1** based on 1,1'-binaphthyl fluorophore. This probe shows high selectivity for sensing Hg by a facile cyclization reaction via fluorescence enhancement in ethanol–HEPES solution. The in situ system from **1** + Hg then exhibited relay recognition for Group IIIA cations via further fluorescence turn-on through the formation of corresponding complexes. Especially, the purified **2** showed high selectivity toward Ga^{3+} over Al^{3+} and In^{3+} by changing the employed media to HEPES buffer (pH = 7.0) solution. To this end, a novel relay recognition from small molecule to cation has been realized via off–on–on fluorescence switch.

This work was supported by National Natural Science Foundation of China (Nos. 21572091 and 21172096).

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