Turn-on Type Chemosensing and Visualization of Hg^{2+} Ions by a Simple NBD Derivative

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The development of selective chemosensors for the detection of biologically important metal ions has received a great deal of attention in recent years.[1] Chemosensors for Hg^{2+} ions are particularly important because mercury and its derivatives are widely used in industry and have extremely toxic impacts on the environment.[2] Although a large number of chemosensors for Hg^{2+} ions have been reported, new functional sensors that can be easily prepared are still necessary to detect various analytes with different matrix and concentration ranges.[3]

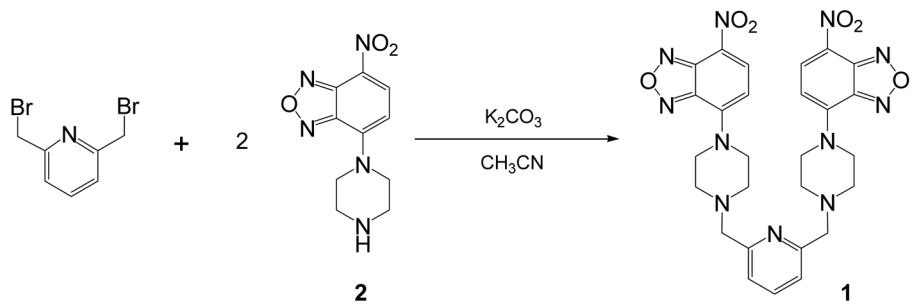
The introduction of two piperazine moieties at the 2,6-position of pyridine provides an efficient binding site in a semi-rigid U-shaped conformation suitable for the recognition of soft metal ions. The binding site has a well defined ligation system comprising five nitrogen atoms: four from piperazine and one from the pyridine ring. Based on this, the molecular framework of the 2,6-bis(aminomethyl)pyridine moiety has been used to prepare efficient signaling systems for transition metal ions.[4] Guo *et al.* have reported an aminonaphthalimide derivative with selective fluorescence enhancement for Hg^{2+} ions that uses the nitrogen atoms of 2,6-bis-(aminomethyl)pyridine as both binding sites as well as quenchers of photoinduced electron transfer (PET).[5] They also reported a very interesting fluorophore system, based on the same U-shaped binding motif, which is capable of a Cu^{2+}-Hg^{2+}-Cu^{2+}-triggered crossword puzzle and logic memory operations.[6]

Hg^{2+} ions are known to be efficient fluorescent quenchers because of enhanced spin-orbit coupling.[7] Therefore, most common fluorescent probes generally undergo nonspecific quenching with Hg^{2+} ions. For this reason, the previously developed chemosensing systems for Hg^{2+} ions mostly exploit the mechanism of complexation-induced fluorescence quenching, and only a few developed probes show fluorescent enhancement with Hg^{2+} ions.[8]

The 7-nitrobenz-2-oxa-1,3-diazole (NBD) subunit is a very attractive moiety for building supramolecular systems to detect a variety of important chemical and biological species, such as Ni^{2+}, Cu^{2+}, Hg^{2+}, Cr^{3+}, and hydroperoxides.[9] These systems are based on the well-known binding motifs of cyclam, crown ether, and acyclic aza-thia compounds. Other interesting NBD-based supramolecular systems are molecular thermometer and nanosensing systems. For example, sensitive fluorescent molecular thermometers were devised based on copolymers of *N*-isopropylacrylamide and NBD-based fluorescent monomers that showed a sharp fluorescence change around 32 °C.[10] Arduini *et al.* reported that NBD-based dye-doped silica nanoparticles and thin films can detect Cu^{2+} ions in the micromolar range.[11] More recently, an NBD derivative with a dipicolylamine binding moiety that exhibits fluorescence probe behavior toward Zn^{2+} ions was developed based on the blocking of PET of the nitrogen atoms.[12] In this paper, we report a simple structured chemosensor, based on a semi-rigid U-shaped conformation, with two convergent NBD subunits for the detection of transition metal ions. The prepared compound exhibited highly selective OFF-ON type fluorescence signaling in aqueous media and enabled efficient visualization of Hg^{2+} ions in living cells.

Compound **1** was prepared by the reaction of 2,6-bis-(bromomethyl)pyridine with NBD-piperazine **2**, which was prepared by the reaction of piperazine with 4-chloro-7-nitrobenzofurazan, in 60% yield.

The chemosensing behavior of **1** was investigated by UV-



2 **1**

Scheme 1

vis and fluorescence measurements. Through a systematic screening of the chemosensing behavior of **1**, a relatively optimized signaling condition of Hepes-buffered aqueous 40% dioxane solution (dioxane:H_2O = 40:60, v/v) was found. The UV-vis spectrum of **1** revealed a characteristic absorption of the NBD unit at 483 nm that was not significantly affected in the presence of various metal ions.

The fluorescence spectra of **1** in aqueous 40% dioxane solution showed a weak and broad fluorescence around 543 nm. In the presence of 100 equiv of various metal ions, the fluorescence intensity significantly increased, particularly with Hg^{2+} ions and somewhat moderately with Cd^{2+} ions (Figure 1). The selective turn-on type signaling behavior of **1** was readily discernible under illumination with a hand-held UV lamp. The fluorescence enhancement efficiency expressed by the ratio I/I_o at 550 nm was 28.7 for Hg^{2+} ions, where I and I_o denote the fluorescence intensity in the presence and absence of metal ions, respectively. With Cd^{2+} ions, a somewhat moderate response was observed (I/I_o = 4.7). Other metal ions had relatively insignificant effects on the fluorescence behavior of **1**, and the efficiency was limited, ranging from 0.61 (Cu^{2+}) to 1.65 (Zn^{2+}). These observations indicate that compound **1** is relatively well optimized for fluorescence sensing of Hg^{2+} ions in an aqueous environment.

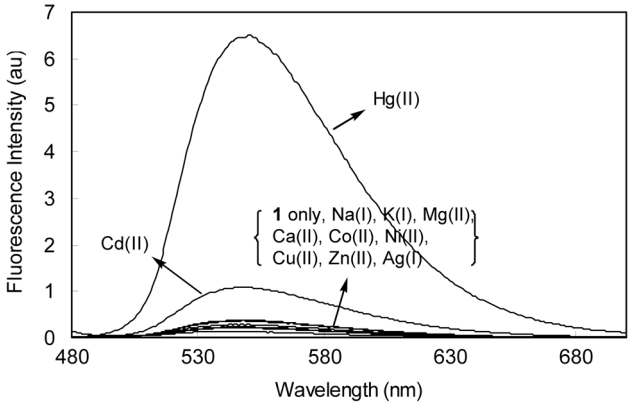


Figure 1. Fluorescence spectra of **1** in the presence of various metal ions in aqueous dioxane solution. [**1**] = 5.0×10^{-6} M, [M^{n+}] = 5.0×10^{-4} M, dioxane:water = 4:6, at pH 7.0 (hepes, 10 mM). λ_{ex} = 460 nm.

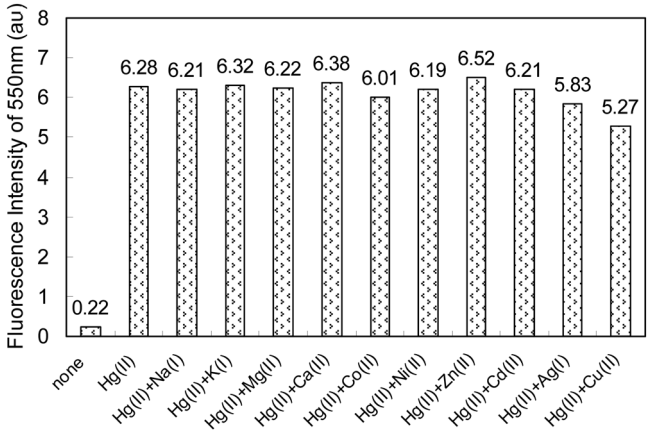


Figure 2. Changes in fluorescence intensity at 550 nm of **1** in the presence of Hg^{2+} ions and various coexistent metal ions. [**1**] = 5.0×10^{-6} M, [Hg^{2+}] = 5.0×10^{-5} M, [M^{n+}] = 5.0×10^{-4} M, dioxane:water = 4:6, at pH 7.0 (hepes, 10 mM). λ_{ex} = 460 nm.

The Hg^{2+}-selective chemosensing behavior of **1** was further investigated by competition experiments employing representative alkali, alkaline earth, and transition metal ions as background. The fluorescence enhancement of **1** induced by the addition of 10 equiv of Hg^{2+} ions was measured in the presence of 100 equiv of coexistent metal ions. Surveyed metal ions exhibited relatively minor interference for Hg^{2+} ion sensing with the exception of Cu^{2+} and Ag^{+} ions (Figure 2).

The quantitative nature of the sensing of Hg^{2+} ions by **1** was elucidated by fluorescence titration of compound **1** in 40% aqueous dioxane solution (Figure 3). As the concentration of Hg^{2+} increased, the fluorescence maximum was slightly red-shifted from 543 to 550 nm. The binding stoichiometry was estimated to be 1:1 by means of a Job's plot. A nonlinear curve fitting of the titration results provides a K_{assoc} value of 1.1×10^6 M^{-1}.[13] Based on the concentration-dependent fluorescence changes, the detection limit was estimated to be 4.7×10^{-7} M. The K_{assoc} and detection limit values obtained are comparable to those obtained with closely related structures that have naphthalimide fluorophores.[5]

The turn-on type signaling is due to suppression of the PET process from the nitrogen atoms of amine to the NBD fluorophore as a result of complex formation. In the present system, the fluorescence-enhancing PET suppression effect overwhelmed the inherent quenching effect exerted by the complexed Hg^{2+} ions.[5,14] The signaling of the compound was reversible, which was confirmed by the interaction with EDTA solution. For example, the fluorescence of **1** was enhanced by the addition of 10 equiv of Hg^{2+} ions (**1** + Hg^{2+}), which decreased to that of **1** itself by subsequent addition of 20 equiv of EDTA solution (**1** + Hg^{2+} + EDTA). Further addition of 20 equiv of Hg^{2+} ions again restored the fluorescence of **1**-Hg^{2+} system, showing the reversibility of

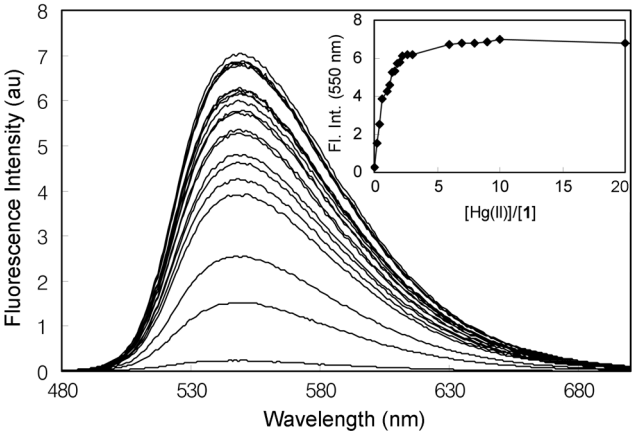


Figure 3. Fluorescence titration of **1** with Hg^{2+} ions. [**1**] = 5.0×10^{-6} M, dioxane:water = 4:6, at pH 7.0 (hepes, 10 mM). λ_{ex} = 460 nm.

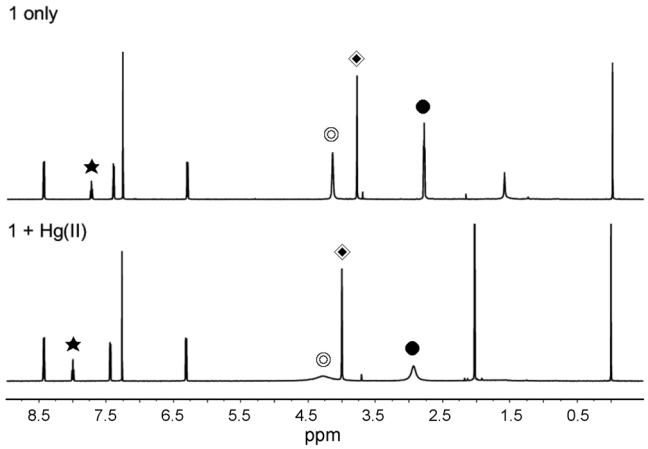


Figure 4. ^{1}H NMR spectra of **1** and **1** in the presence of Hg^{2+} ions. [**1**] = 2.0×10^{-2} M, [$Hg(OAc)_2$] = 3.0×10^{-2} M. DMSO-d_6.

the Hg^{2+}-selective chemosensing behavior of compound **1**.

The complex formation was confirmed by NMR measurements. Upon treatment with Hg^{2+} ions, a significant shift from δ 7.73 to 8.00 ppm was observed for 4-H proton of pyridine moiety in the ^{1}H NMR spectra of **1** (Figure 4). The methylene protons adjacent to the pyridine moiety also experienced moderate downfield shifts from δ 3.79 to 3.99 ppm. Other noticeable changes were the piperazine protons, which also experienced moderate downfield shifts from δ 2.80 to 2.93 ppm and δ 4.15 to 4.28 ppm with significant broadening. Changes in ^{13}C NMR spectra also suggested the formation of a complex of **1** and Hg^{2+} ions by revealing considerable shifts from δ 157. 7 to 154.1 (*C*-2, Pyr), 137.6 to 141.3 (*C*-4, Pyr), 121.6 to 125.6 (*C*-7, NBD), and 63.4 to 61.3 ppm (N*C*H_2-Pyr).

Lastly, we evaluated the turn-on type Hg^{2+}-signaling ability of **1** within living cells.[15] As shown in Figure 5A, PC12 cells incubated with compound **1** (10 μM) for 90 min at 37 °C show no intracellular fluorescence. However, when cells treated with **1** were further incubated with 100 μM $HgCl_2$ in the growth medium for 40 min, marked increases in the fluorescence intensity of cells were observed (Figure 5B). This result suggests that **1** has moderate cell permeability and can be used to image intracellular Hg^{2+} ions in living cells.

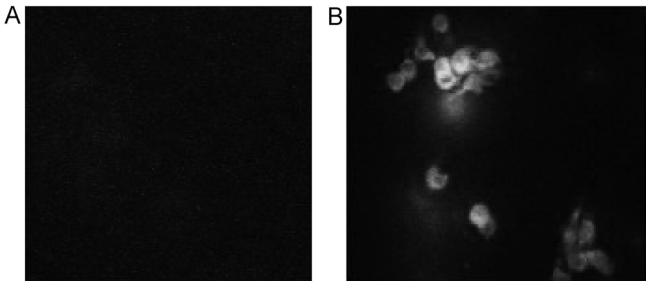


Figure 5. Confocal fluorescence images of Hg^{2+} ions in PC12 cells. (A) Fluorescence image of cells incubated with **1** (10 μM). (B) Fluorescence image of cells incubated with **1** (10 μM) for 90 min at 37 °C, washed three times, and then further incubated with 100 μM $HgCl_2$ for 40 min at 37 °C.

In summary, we prepared a new, simple-structured chemosensor by combining an NDB moiety as a signaling unit and a 2,6-pyridyl moiety as a convergent binding backbone. The chemosensor exhibited a selective OFF-ON type response toward Hg^{2+} ions in the micromolar range over other coexistent transition metal ions in aqueous dioxane solution. The compound also enabled fluorescent visualization of Hg^{2+} ions in living cells.

Experimental Section

General. 2,6-Bis(bromomethyl)pyridine, piperazine, and 4-chloro-7-nitrobenzofurazan were purchased from Aldrich Chemical Co. and used without further purification. ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectra were obtained on a Varian Gemini-2000 spectrometer. UV-vis spectra were recorded with a Jasco V-550 spectrophotometer. Fluorescence spectra were measured on an Aminco-Bowman Series 2 Spectrophotometer. All solvents used for the measurements of UV-vis and fluorescence spectra were purchased from Aldrich Chemical Co. as 'spectroscopic grade'. Mass spectral data were obtained with a Micromass Autospec mass spectrometer. NBD-piperazine **2** was prepared by the reaction of piperazine with 4-chloro-7-nitrobenzofurazan following the reported procedure.[16]

Synthesis of 1. A mixture of 2,6-bis(bromomethyl)-pyridine (53 mg, 0.2 mmol), NBD-piperazine (112 mg, 0.45 mmol), and K_2CO_3 (83 mg, 0.3 mmol) in acetonitrile was refluxed under N_2 atmosphere. After 24 h of reaction, the reaction mixture was evaporated and the residue was partitioned between CH_2Cl_2 and water. The organic layer was separated and evaporated under reduced pressure. The crude product was purified by the column chromatography (silica gel, CH_2Cl_2-MeOH) to yield orange colored product. Yield, 60%. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.47 (d, J = 9.0 Hz, 2H), 7.81 (t, J = 7.7 Hz, 1H), 7.41 (d, J = 7.8 Hz, 2H), 6.65 (d, J = 9.0 Hz, 2H), 4.15 (br m, 8H), 3.69 (s, 4H), 2.70 (t, J = 4.8 Hz, 8H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 157.7, 145.7, 145.3, 145.2, 137.6, 136.7, 121.8, 121.6, 104.0, 63.4, 52.8, 49.8. MS (FAB, *m*-NBA) $C_{27}H_{28}N_{11}O_6$ $[M+1]^+$, Calcd, 602.2. Found, 602.1.

PC12 Cell Culture and Fluorescence Imaging Experiments. PC12 cells were cultured in RPMI-1640 medium supplemented with 10% horse serum, 5% fetal calf serum, 100 IU/mL penicillin, 100 μg/mL streptomycin, 250 ng/mL amphotericin B, and 100 μg/mL kanamycin sulphate (all from Gibco). One day before imaging, cells were passed and plated on glass coverslips coated with poly-L-lysine (50 μg/mL, Sigma). Immediately before the experiments, cells were washed with PBS buffer, incubated with the probe in PBS, and imaged. Confocal fluorescence imaging was performed with a Zeiss LSM510 META laser scanning microscope containing an Axioplan 2 MOT upright microscope. Excitation of **1**-loaded cells at 450 nm was carried out, and emission was collected at 550 nm using a META detection system.

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