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Reaction-Based Two-Photon Fluorescent Probe for Turn-On Mercury(II) Sensing and Imaging in Live Cells

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Among the factors that cause environmental problems, heavy-metal ions have gradually become the focus of increasing concern. Mercury(II) ions, which are a typical contaminant, are enormously toxic to the human body and other organisms, even at low concentrations, and they bioaccumulate through the food chain.[1] Various diseases and biofunctional disorders related to the brain, heart, kidneys, stomach, intestines, and other organs are caused by Hg^{II} contamination, which makes necessary the detection of trace amounts of Hg^{II} ions in environmental and biological samples.[2]

During the past decade, various Hg^{II} sensors utilizing colorimetric and fluorescent methods, including small molecules, DNA, nanoparticles, polymers, and bio-macromolecules, have been developed.[3] Many of these systems are limited by onerous synthetic procedures or low water solubility, which restrict further applications. Another limitation is that most of these one-photon fluorescent probes require high-energy excitation and have relatively low signal/noise ratios.[4] Compared to their one-photon counterparts, two-photon (TP) fluorescent probes have generated greater interest due to their lower excitation energy, increased penetration depth (>500 nm), and greatly reduced tissue autofluorescence and self-absorption.[5] Besides, most of these probes can only detect inorganic Hg^{II}, and those that sense methylmercury, which is notoriously poisonous, are rare.[6] Hence, the development of TP fluorescence sensors for inorganic mercury ion and methylmercury detection in live cellular environments is a critical challenge in biological chemistry.

Novel chemical probes with great selectivity and sensitivity towards target analytes[7] have been developed through the introduction of unique reactive groups into latent fluorophores.

On the basis of the mercury desulfurization reaction, we have designed a turn-on two-photon fluorescent probe, 6-(2-methyl-1,3-dithian)-*N*-methyl-2-naphthylamine (SAN), for Hg^{2+} sensing. SAN was synthesized in high yield (Scheme 1b) by a straightforward protection reaction of 6-acyl-*N*-methyl-2-naphthylamine (AAN) with 1, 3-propanedithiol. The strong thiophilicity of Hg^{2+} selectively promotes the deprotection of the thioketal group of SAN to the original acyl group of AAN. The product AAN is highly fluorescent due to its one-photon and two-photon absorption properties.[8] The signaling mechanism is believed to be an intramolecular charge transfer (ICT) effect between the electron-donor methylamino group and the electron-acceptor acyl group in AAN, while in the case of SAN, the ICT effect is blocked to a certain extent.[9] Additionally, the change in fluorescent emissions for the entire process can be observed visually (Scheme 1a).

The feasibility of the Hg^{2+} sensing method was investigated using emission spectroscopy. As shown in Figure 1a, the one-photon fluorescence of SAN, which was weak initially, displayed a significant change after incubation with Hg^{2+} under simulated physiological conditions (phosphate-buffered saline (PBS), pH 7.4) for 24 h. As the concentration of Hg^{2+} increased, the emission band centered at 503 nm progressively increased and showed a nearly 230-fold enhancement. A similar consistent change was observed in the two-photon fluorescence spectrum of SAN upon addition of excess Hg^{2+} (Figure S2 in the Supporting Information). The one-photon fluorescent enhancement exhibited a linear relationship ($R=0.995$) with Hg^{2+} concentration up to 3 μM (Figure 1b), with a detection limit of 26 nM, thus indicating that SAN is suitable for detecting Hg^{2+} in the parts-per-million concentration range. In addition, SAN is pH-insensitive, which means that it has a strong response toward Hg^{2+} at biologically relevant pH values (pH 4–9); hence, it can be adapted for Hg^{2+} sensing in biotic environments (Figure 2). This mercury-ion-promoted desulfurization reaction is also

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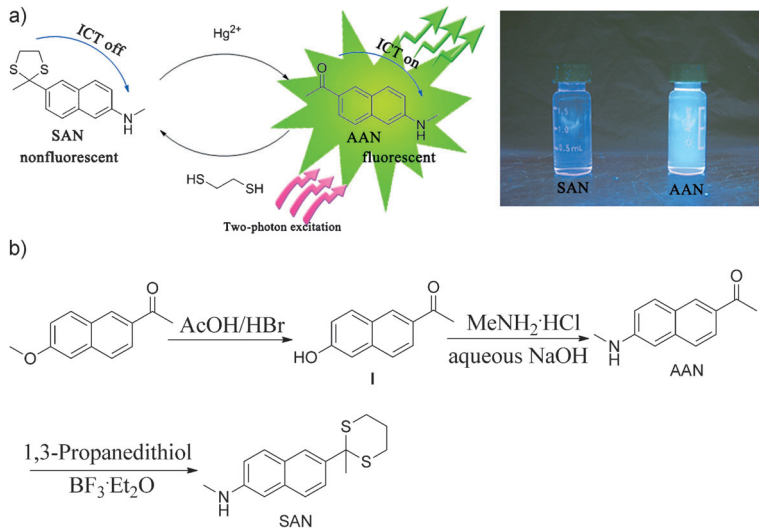

Scheme 1. a) Hg^{2+} sensing process of SAN and the photographs of SAN before and after the addition of Hg^{2+}. b) Synthesis of Probe SAN.

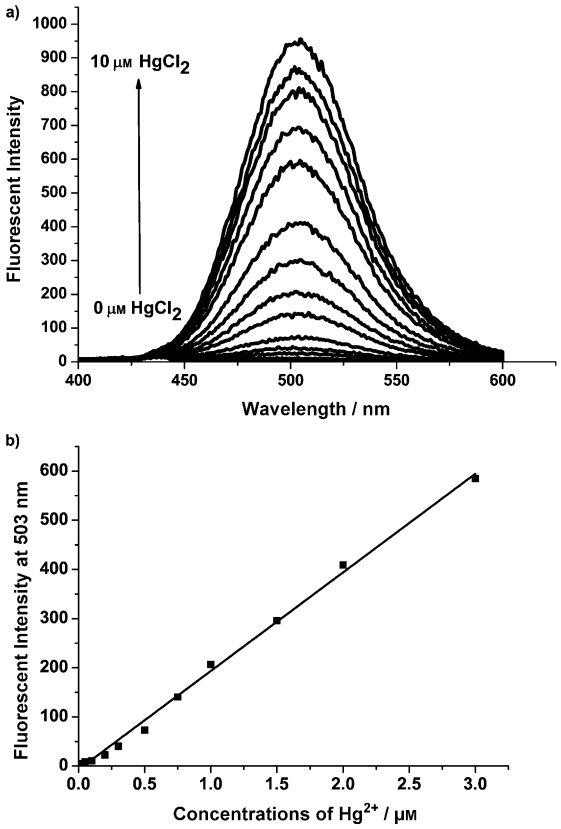

Figure 1. a) One-photon fluorescent titration curves of SAN(3 μM) in the absence and presence of Hg^{2+}. $HgCl_2$ concentration ranges from 10 nM to 10 μM in PBS buffer (pH 7.4). The excitation wavelength was 380 nm. b) Plot of the fluorescent intensity at 503 nm against Hg^{2+} concentration (0–3 μM).

anticipated to occur with methylmercury species (CH_3HgX). In Figure 3, SAN displayed modest fluorescence recovery upon treatment with CH_3HgCl for 24 h in the PBS buffer.

The Hg^{2+} selectivity of our probe SAN was evaluated in comparison with thirteen other metal ions under similar conditions (Figure 4). The reaction between SAN and Hg^{2+} resulted in a dramatic fluorescent enhancement; while the solutions of Pb^{2+} (100 μM) and Ag^{+} (20 μM) showed negligible changes in fluorescence properties. The solutions of other metal cations (100 μM Cd^{2+}, Li^{+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Na^{+}, K^{+}, Ca^{2+}, Co^{2+}, Cu^{2+}, and Zn^{2+}) showed no emission change. Furthermore, as illustrated in Figure S3 in the Supporting In-

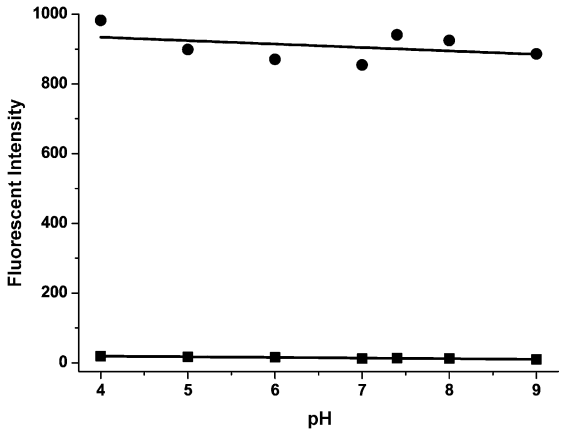

Figure 2. Effect of pH value on the one-photon fluorescence intensity of the reaction product between SAN (3 μM) and Hg^{2+} (5 μM). The reaction was carried out for 24 h at 37 °C in 10 mM phosphate buffer. The emission intensity at 503 nm was recorded. ■ SAN only, ● samples in the presence of Hg^{2+}.

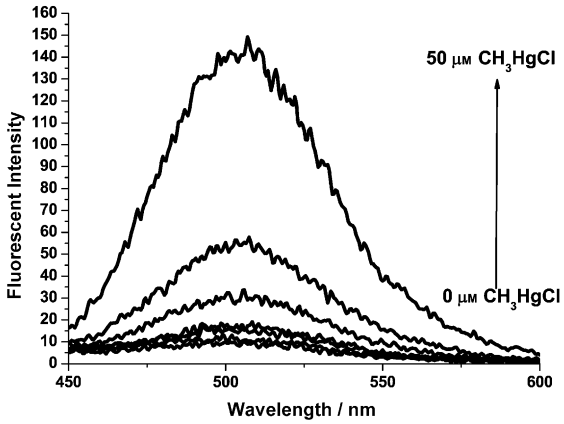

Figure 3. One-photon fluorescent titration curves of SAN (3 μM) in the absence and presence of methylmercury. CH_3HgCl concentration ranges from 0 to 50 μM in PBS buffer (pH 7.4). The excitation wavelength was 380 nm and incubation time is 24 h.

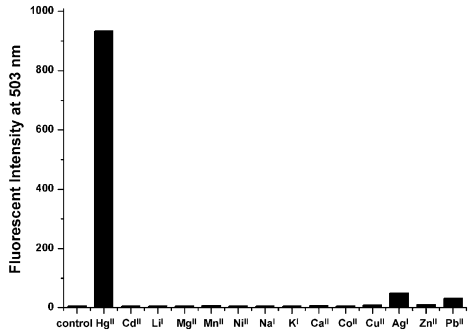

Figure 4. Selectivity studies. Fluorescent responses of SAN toward Hg^{2+} and 13 different metal ions (Cd^{2+}, Li^{+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Na^{+}, K^{+}, Ca^{2+}, Co^{2+}, Cu^{2+}, Ag^{+}, Zn^{2+} and Pb^{2+}) at 503 nm. c(SAN) = 3 μM, c-(Hg^{2+}) = 5 μM, c(Ag^{+}) = 20 μM, and c(other cations) = 100 μM in PBS buffer (pH 7.4).

formation, an unperturbed fluorescence response was observed even when Hg^{2+} was added to the mixture of various competing ions, each at a concentration of 50 μM. All these results undisputedly confirmed that SAN demonstrated an excellent specificity and selectivity towards Hg^{2+} ions.

To demonstrate the formation of AAN and gain further insights into the mechanism of Hg^{2+} sensing, two experiments were performed. SAN was treated with Hg^{2+} ions for 0.5 h, and the reaction product was purified using column chromatography. The ^{1}H NMR spectrum of isolated product identified it as AAN (Figure S1b). We also investigated the UV/Vis absorbance change during the reaction. Addition of Hg^{2+} ions to the SAN solution resulted in a red shift of the maximum absorption wavelength, from approximately 295 to 355 nm, in accordance with the pull–push ICT effect of the two-photon product (AAN; Figure S4 in the Supporting Information). Based on these results, it is conceivable that Hg^{2+} converts the protected thioketal group to the acyl group, leading to the formation of AAN and fluorescence turn-on.

We next sought to utilize SAN as a TP probe for Hg^{2+} detection in live-cell environments (Figure 5). The TPM image of the HeLa cells labeled with 5 μM SAN at 37 °C showed minimal background emission. However, the TP fluorescence increased significantly when the cells were exposed to 50 μM Hg^{2+} for 3 h. The bright-field image confirmed cell viability during the imaging experiments. Hence, SAN can be used as a probe for sensing Hg^{2+} in live cells.

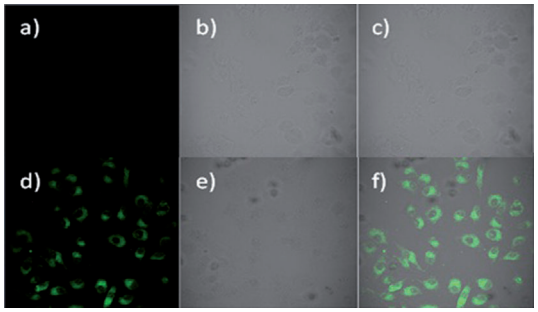

Figure 5. Two-photon microscopy. TPM images (a, d), bright-field images (b, e), overlay of TPM and bright-field images (c, f) of HeLa cells. HeLa cells were labeled with a) SAN (5 μM) for 4 h, d) SAN (5 μM) for 1 h and subsequently treated with Hg^{2+} (50 μM) for 3 h. The TP fluorescence was collected at 500–550 nm upon excitation at 780 nm. Cells shown are representative images from replicate experiments (n = 4).

In conclusion, we have developed a Hg^{2+}-promoted desulfurization reaction for Hg^{II} detection by employing a turn-on two-photon fluorescent probe SAN. Our approach is potentially suitable for inorganic and organic Hg^{II} sensing with high specificity from the nanomole to the micromole scale and live cell imaging. Using the advantages of two-photon fluorescence, it should be possible to detect trace amounts of Hg^{II} in live tissues.

Experimental Section

Optical Properties Study

One-photon fluorescent emission spectra were collected from 400–600 nm on a PerkinElmer LS 55 instrument with an excitation wavelength of 380 nm; the excitation and emission slit widths were both 4 nm. The sample were prepared by mixing SAN, buffer, deionized water, and $HgCl_2$ or CH_3HgCl with given concentrations to final volume 2 mL. Then the samples were incubation at 37 °C for 24 h. Quartz cuvettes with 2 mL volume were used for emission measurements. For two-photon excitation experiments, all samples were excited at 760 nm by a mode-locked Ti:-Sapphire femtosecond pulsed laser (Chameleon Ultra I, Coherent Inc.) with a pulse width of 140 fs at a repetition rate of 80 MHz. Photoluminescence was recorded on a DCS200PC Photon Counting with single-photon sensitivity through an Omni-λ5008 monochromator (Beijing Zolix Instruments Co., Ltd). UV/Vis absorption spectra were collected on a SHIMADZU UV-2550 spectrophotometer from 200 to 700 nm with 600 μL quartz cuvettes. Unless otherwise specified, all spectra were taken at an ambient temperature in 10 mM phosphate-buffered saline (PBS) at pH 7.4.

Selectivity Experiments

The Hg^{2+} selectivity of SAN was evaluated in comparison with thirteen other metal ions (Cd^{2+}, Li^{+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Na^{+}, K^{+}, Ca^{2+}, Co^{2+}, Cu^{2+}, Pb^{2+}, and Zn^{2+}) under similar conditions. Fifteen samples with or without different metal ions were incubated at 37 °C for 24 h. The fluorescent emission intensity at 503 nm was used to plot a histogram. For mixture samples containing various competing ions, each at a concentration of 50 μM, fluorescent response was detected in the absence or presence of Hg^{2+}.

Cell Culture and TPM Imaging

HeLa human cervical carcinoma cells (CCTCC, China) were cultured in Dulbecco's modified Eagle's medium (DMEM, Hyclone, China) supplemented with 10% fetal bovine serum (FBS, Hyclone), penicillin (100 units mL^{-1}), and streptomycin (100 μg mL^{-1}). All the cells were maintained in a humidified atmosphere of 5:95 (v/v) of CO_2/air at 37 °C. One day before imaging, the cells were passed and plated on glass-bottomed dishes (Nest). For labeling, the growth medium was removed and replaced with DMEM without FBS. The cells were incubated with 3 μM SAN for 1 h at 37 °C and were washed three times with DMEM without FBS. Then 50 μM $HgCl_2$ was added to the cells, which were imaged after 3 h. As a control, another dish of cells was incubated with 3 μM SAN for 1 h at 37 °C and then incubated with DMEM for 3 h. Two-photon fluorescence microscopy images of SAN-labeled cells and tissues were obtained with spectral confocal and multiphoton microscopes (Zesis LSM 710NLO) with a ×40 (NA = 0.30 DRY) objective lens. To obtain images at 500–550 nm, internal photomultiplier tubes were used to collect the signals in 8-bit unsigned 1024 × 1024 pixels at 400 Hz scan speed.

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Keywords: fluorescent probes · mercury · microscopy · sensors

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