Fluorescent detection of methylmercury by desulfurization reaction of rhodamine hydrazide derivatives†

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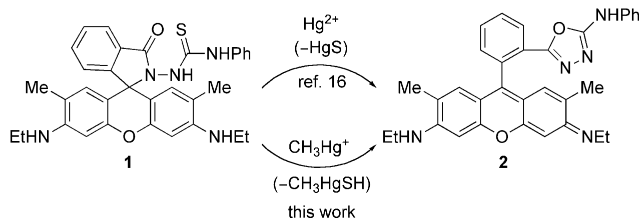
Exposure to methylmercury causes severe damage to various tissues and organs in humans. Although a variety of fluorescent chemosensors have been exploited, only few biological monitoring systems for organomercury species have been described to date. In this report, we describe an irreversible rhodamine chemosensor for the detection of methylmercury and real-time monitoring of methylmercury in living cells and organisms.

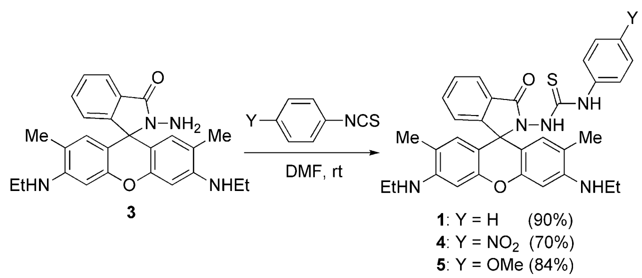
Mercury exists in the environment as inorganic mercury species (Hg^0, Hg^{2+}) and organic mercury (*e.g.* RHg^+, R_2Hg, where R = typically Me, Et).[1] Methylation of inorganic mercury species by aquatic microorganisms can produce methylmercury compounds.[2] The biological targets and toxicity profile of mercury species depend on their chemical composition.[3] Methylmercury species (CH_3HgX), which can readily pass through biological membranes,[4] are powerful neurotoxicants[5] to fish, animals, and humans.[6] Neurological damages[7] associated with methylmercury intoxication are manifold and include prenatal brain damage, cognitive and motion disorders, vision and hearing loss, and Minamata disease.[8] Several targets, such as the BBB (blood brain barrier), axonal transport, neurotransmission, synthesis of protein, DNA, and RNA, have been proposed as sites sensitive to methylmercury.[9] The ramifications of long-term or short-term and low-level exposure to methylmercury are less clear and warrant thorough toxicological investigations. Therefore, the biological effects of methylmercury species have been considerably studied.[10]

Fluorescent sensors based on small molecules,[11] polymeric materials,[12] nanoparticles,[13] and dosimeters[14] have served as tracing tools for neurotoxic Hg^{2+}. Recently, the sensing and imaging of Hg^{2+} in living biological systems have also been realized.[15,16] Fluorescent detection of methylmercury in the environment and biological systems using chemosensor techniques has not been studied well. Recently, Ahn's group has reported a fluorescein-based vinyl ether probe for detection of Hg^{2+} and MeHgX.[17] They utilized fluorescence changes associated with methylmercury-promoted hydrolysis of vinyl ether. With this "turn-on"-type green fluorescent probe, they also demonstrated imaging of cells and zebrafish. From the previous experiments on the Hg^{2+}-selective chemosensor utilizing the Hg^{2+}-induced desulfurization reaction (Scheme 1),[16] we observed that a similar desulfurization reaction could be promoted by CH_3Hg^+ too, but less efficiently. Although CH_3Hg^+ is less thiophilic than Hg^{2+}, we expected the same rhodamine hydrazide system could serve as a CH_3Hg^+ probe too. Herein, we report our results on the fluorescent sensing of methylmercury in aqueous solutions and applications to biological imaging by using the rhodamine thiosemicarbazides.



Scheme 1 Hg^{2+}-induced desulfurization of rhodamine thiosemicarbazide **1**.

In addition to the previously reported rhodamine thiosemicarbazide **1**, we also prepared rhodamine thiosemicarbazide derivatives containing *p*-NO_2 (**4**) and *p*-OMe (**5**) groups to see the reactivity differences caused by the electron density on the aniline ring. The probes were synthesized from the known rhodamine 6G hydrazide **3** and aryl isothiocyanates in good yields (Scheme 2).



Scheme 2 Synthesis of rhodamine thiosemicarbazides.

Upon addition of CH_3Hg^+ to solutions (10 μM) of the rhodamine thiosemicarbazides (**1**, **4**, or **5**) in water (DMSO 1%) at 25 °C, the fluorescence intensities (at 560 nm) of the solutions increased gradually as shown in Fig. 1. While the probe **1** showed strong fluorescence intensity changes (Fig. 1A), the rhodamine derivatives with an electron withdrawing group (**4**) or an electron donating group (**5**) exhibited weak fluorescence intensity changes under the same conditions (Fig. 1C, D). As expected, probe **1** required greater amounts of CH_3Hg^+ (needs 6–8 equiv., Fig. 1B) than Hg^{2+} (needs 1 equiv.)[16a] for the saturation of the fluorescence intensity.

The detection limit of probe **1** for CH_3Hg^+ was evaluated by monitoring the fluorescence titration curves of **1** (10^{-6} M) with CH_3Hg^+ at nanomolar (nM) levels (Fig. 2). The fluorescence intensity of **1** was nearly proportional to the amount of CH_3Hg^+ added (Fig. 2B) and detection was possible at 200 nM of CH_3Hg^+ in water (DMSO 1%) at 25 °C. The fluorescence response of **1** to

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† Electronic supplementary information (ESI) available: Experimental and copies of ^{1}H and ^{13}C NMR spectra. See DOI: 10.1039/b915723a

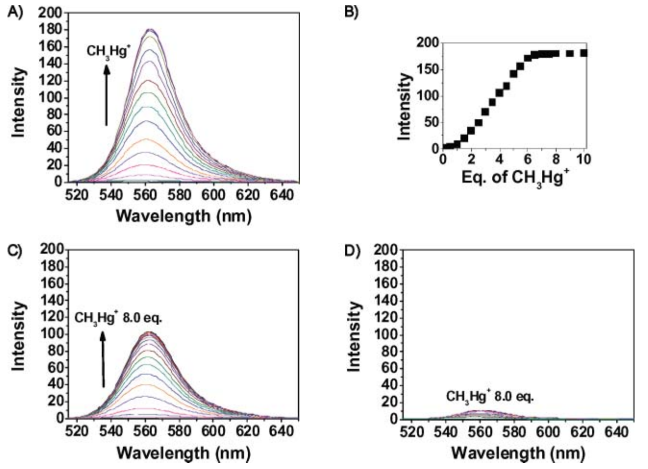


Fig. 1 Fluorescence titration curves of the chemosensors with methylmercury. Fluorescence responses of **1** (A), **4** (C), and **5** (D) (10 μM) upon additions of CH_3Hg^+ (0–8.0 equiv.) in water (DMSO 1%) at 25 °C (excitation at 500 nm; emission at 560 nm). Each spectrum was acquired 5 min after each addition of CH_3Hg^+. (B): Plot of fluorescence intensity (at 560 nm) *versus* equivalents of CH_3Hg^+ shown in the titration curve A.

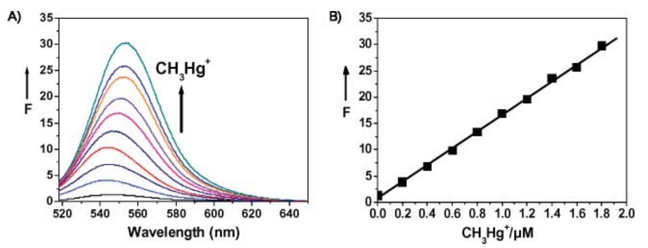
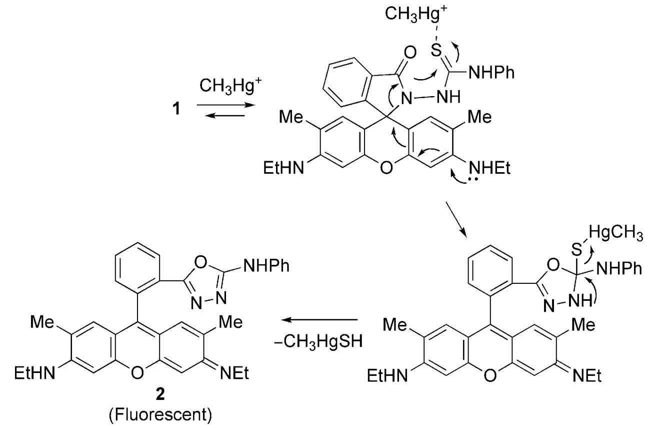


Fig. 2 (A) Fluorescence emission changes of **1** (10^{-6} M) upon additions of CH_3Hg^+ (by 200 nM) in water (DMSO 1%) at 25 °C. (B) The fluorescence intensity changes at 550 nm (excitation at 500 nm) *versus* the concentration of CH_3Hg^+.

CH_3Hg^+ in PBS-buffer solutions (DMSO 1%, at pH 7.4) showed similar trends to those observed in aqueous solutions (DMSO 1%). Reaction of **1** with CH_3Hg^+ is slow and requires an excess of CH_3Hg^+ for completion of the reaction. Typically, ~10 equiv. of CH_3Hg^+ is required to meet the fluorescence enhancement induced by 1 equiv. of Hg^{2+} in the given reaction time period (see ESI†).

The fluorescent product obtained from the reaction of **1** with CH_3HgCl proved to be the 1,3,4-oxadiazole compound **2** which is observed from the reaction **1** with $HgCl_2$.[16] Therefore, a similar desulfurization reaction mechanism could be responsible for the fluorescent detection of methylmercury by **1**. In this case, CH_3HgSH could be eliminated as the result of the desulfurization reaction as proposed in Scheme 3.

We then evaluated bio-imaging applications of **1** for detection of organomercury species in biological systems. HeLa cells were incubated with 20 μM of **1** for 10 min at 37 °C, washed with PBS-buffer (pH 7.4) to remove the remaining chemosensors, then the treated cells were incubated with 5–20 μM of CH_3HgCl in culture medium for 10 min at 37 °C. While the HeLa cells treated with only **1** did not show any fluorescence (Fig. 3e), the HeLa cells treated with both **1** and CH_3HgCl displayed strong fluorescence intensity (Fig. 3f–h). The microscopic and fluorescent images clearly indicate that probe **1** can detect 5–20 μM of methylmercury



Scheme 3 A proposed mechanism for the CH_3Hg^+-induced desulfurization reaction of **1**.

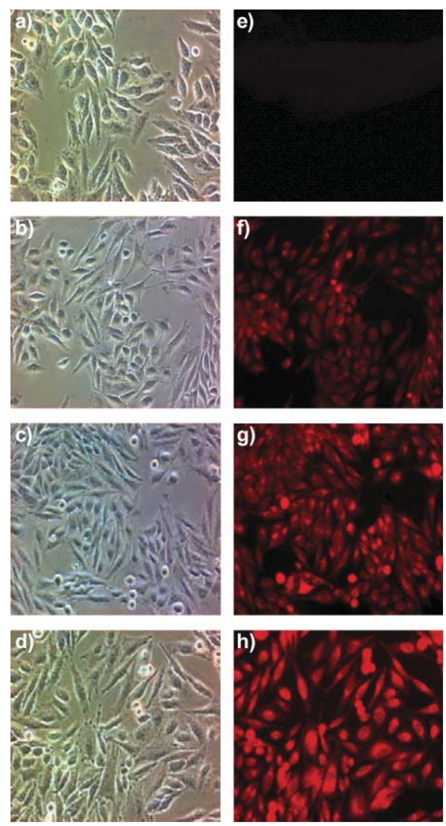


Fig. 3 Fluorescence images of methylmercury in live HeLa cells. Microscopic (a) and fluorescence (e) images of HeLa cells treated with **1** (20 μM) in the absence of CH_3Hg^+. Microscopic (b–d) and fluorescence (f–h) images of HeLa cells treated with both CH_3Hg^+ (b, f—5 μM, c, g—10 μM, d, h—20 μM) and **1** (20 μM).

in live HeLa cells. We were able to detect 300 nM of CH_3HgCl in HeLa cells by this method (see ESI†).

Next, time-dependent uptake of methylmercury in live cells was determined by incubating cells with probe **1** and methylmercury while measuring the fluorescence intensity changes as a function of time. The HeLa cells and A549 cells were incubated with **1** (20 μM) for 30 min, washed with PBS to remove the remaining sensors, then treated with 0–200 μM of CH_3HgCl in the culture media. The fluorescence intensity changes were continuously monitored by using a fluorescence microplate reader. Although full saturations of the fluorescence intensity were not observed in the live cell uptake experiments, methylmercury can clearly enter the cells within 30–40 min as shown in Fig. 4.

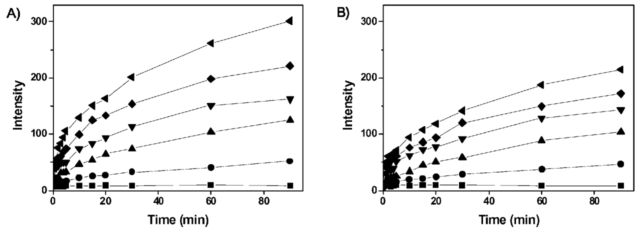


Fig. 4 Real-time monitoring of methylmercury uptake in live cells. Real-time monitoring of CH_3Hg^+ (0 (■), 10 (●), 20 (▲), 40 (▼), 100 (◆), and 200 (◀) μM) uptakes by (A) HeLa cells and (B) A549 cells using **1** (20 μM). The fluorescence intensity changes in the cells were continuously monitored by a fluorescence microplate reader (excitation at 500 nm, emission at 560 nm).

Encouraged by the live cell experiments, we examined if chemosensor **1** could be used to detect methylmercury in living organisms. Four-day old zebrafish was first incubated with 20 μM of **1** for 30 min at 28 °C and then exposed to 20 μM of CH_3HgCl for 10 min after removal of the remaining chemosensor. While the zebrafish treated with only probe **1** did not show any fluorescence (Fig. 5c, d), the zebrafish treated with both CH_3HgCl and **1** displayed strong red fluorescence (Fig. 5e, f). Interestingly, strong fluorescence intensity was observed in the eye lens and liver regions as shown in Fig. 5e, f.[18] We were able to image zebrafish[19] incubated in 100 nM CH_3HgCl media without any problems by this method (see ESI†).

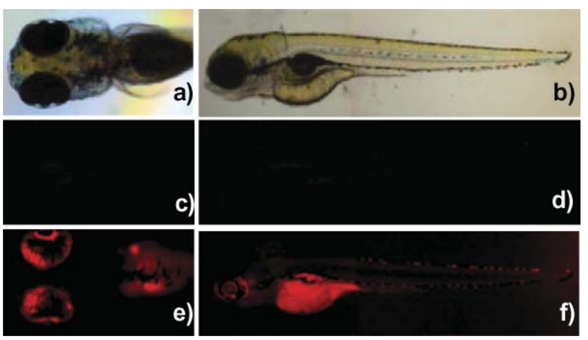


Fig. 5 Microscopic and fluorescent images of zebrafish. The 4-day old zebrafish was treated with probe **1** (20 μM) for 30 min, washed with PBS-buffer to remove the remaining chemosensors, and incubated with CH_3HgCl for 10 min. Dorsal (a, c, e) and lateral views (b, d, f); (a, b) microscopic images of zebrafish treated with probe **1** and CH_3HgCl (20 μM). (c, d) Fluorescent images of zebrafish treated with probe **1** in the absence of CH_3HgCl. (e, f) Fluorescent images of zebrafish treated with probe **1** and CH_3HgCl.

In summary, we have described an irreversible chemosensor for the detection of methylmercury species in live cells and zebrafish. The rhodamine thiosemicarbazide probe, which reacts irreversibly with methylmercury *via* a desulfurization reaction, can detect methylmercury with high sensitivity in aqueous media. Fluorescent imaging of HeLa cells and zebrafish successfully demonstrated the detection of methylmercury in living cells and organisms.

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