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Rosamine-Based Fluorescent Sensor with Femtomolar Affinity for the Reversible Detection of a Mercury Ion

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Supporting Information

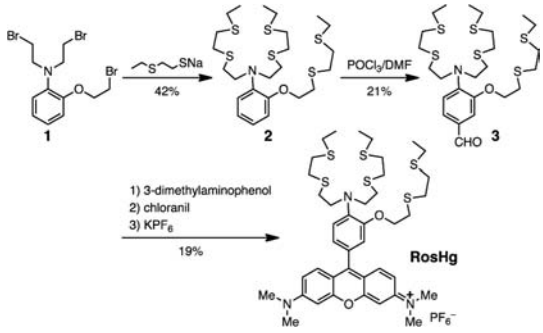
ABSTRACT: A Hg^{2+}-selective fluorescent sensor, RosHg, has been developed based on a rosamine platform. RosHg exhibited a ~20-fold increase in fluorescence emission upon binding with Hg^{2+}, and the enhanced fluorescence was immediately decreased when glutathione was added to a solution of the Hg-RosHg complex. The dissociation constant for the Hg^{2+} complex was determined to be 0.10 fM by using a set of Hg^{2+}/Mg^{2+}/ethylenediaminetetraacetic acid buffer solutions. Confocal microscopy experiments demonstrated that this sensor can monitor changes of the Hg^{2+} level in the mitochondria of living cells.

Heavy-metal pollution triggers serious environmental disasters and health problems.[1] Mercury is recognized as a highly poisonous neurological toxin.[2] In particular, fetuses and children are very susceptible to the effects of mercury, and even low concentrations of mercury exposure during the early stages of development, for example, by consumption of fish and seafood, can result in a serious neural disorder such as Minamata disease.[3] Mercury pollution caused by gold mining has also become more acute in developing countries and is associated with wide-reaching health hazards.[4]

Fluorescent sensors that enable the detection and quantification of heavy-metal ions are an important tool in the field of molecular biology for studying the details of metal functionality or toxicity at the molecular level, as well as in environmental studies.[5] Many fluorescent sensors based on small molecules,[6−8] short peptides,[9] oligonucleotides,[10] and gold nanoparticles[11] have been developed; some of these are useful for sensing Hg^{2+} in cells and vertebrate organisms such as fish.[7,8] Generally, Hg^{2+} binding results in fluorescence quenching because of the effective intersystem crossing process to the triplet state of the fluorophore by the heavy-atom effect. Thus, the design of fluorescent sensors for Hg^{2+} is based on oxymercuration or mercury-promoted desulfurization, by which a nonfluorescent molecule is converted into the corresponding fluorescent product.[12] However, in these cases, it is often necessary for the reaction time to exceed 5 min to reach the maximum fluorescence intensity and, consequently, they are unsuitable for monitoring intracellular Hg^{2+} behavior in real time. Moreover, these sensors cannot detect the change of free Hg^{2+} levels associated with intracellular binding and/or cellular elimination of Hg^{2+}. To date, considerable efforts have been focused on the development of fluorescent sensors with the capacity of a reversible off−on response to Hg^{2+}; however, there are few examples that have exhibited a reversible response to Hg^{2+} in biological samples.[8]

We have previously reported the Ag^{+}-selective fluorescent sensor RosAg with a tetramethylrosamine scaffold, where *o*-aminophenol bearing three thioether groups as the metal-ion receptor was directly introduced into the xanthene core.[13] The fluorescence of RosAg was efficiently quenched ($\Phi < 0.005$) by photoinduced electron transfer (PET), whereas a significantly enhanced emission was observed upon Ag^{+} binding, but not Hg^{2+}. Using RosAg as a template, we have developed a new fluorescent sensor, RosHg, for the reversible detection of Hg^{2+} (Scheme 1). This sensor contains a hexathioether moiety in the metal binding site, which is expected to preferably bind to soft metal ions including Hg^{2+} over hard metal ions such as Ca^{2+} and Zn^{2+}.[6a,13,14] RosHg exhibits a rapid increase in the fluorescence emission intensity upon Hg^{2+} binding with an extremely high binding affinity in the range of $K_d = 0.1$ fM. Confocal microscopy experiments demonstrated that this sensor can localize to mitochondria and probe changes of free Hg^{2+} levels in living cells.

Scheme 1. Synthesis of RosHg



The synthesis of RosHg is outlined in Scheme 1. The reaction of tribromide **1** with 3-thia-1-pentanethiol[14a] produced hexathioether ligand **2**, which is a newly designed Hg^{2+}-selective receptor. Formylation of **2** under Vilsmeier conditions followed by a Friedel−Crafts reaction with 3-(dimethylamino)phenol, chloranil oxidation, and an anion-exchange reaction with KPF_6 afforded the desired sensor RosHg.

In order to gain insight into the binding profiles of RosHg and Hg^{2+}, we initially compared the ^1H NMR spectra of truncated thioether ligand **2** and its Hg^{2+} complex. When $HgCl_2$ was added to a solution of thioether ligand **2** in dimethyl sulfoxide

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(DMSO)-d_6, the signals of the methylene protons adjacent to the sulfur atoms were shifted more downfield (Δppm = 0.2−0.4) than those of the terminal methyl protons (Δppm ~ 0.05; Figure S1 in the Supporting Information, SI). This indicates that all sulfur atoms are coordinated in the first coordination sphere of the Hg^{2+} complex in the solution. It should be noted that the downfield shifts of the aromatic protons for the Hg^{2+}-RosHg complex were somewhat smaller compared with those for the Ag^{+}-RosAg complex,[13] suggesting a weaker metal−nitrogen interaction in the Hg^{2+}-RosHg complex. This may affect the inhibition efficiency of the fluorescence quenching via PET.

All spectroscopic measurements for RosHg were performed in a mixture of 50 mM HEPES (pH 7.20, 0.1 M KNO_3) and DMSO (4:1, v/v).[15] In the absence of Hg^{2+}, RosHg exhibited a characteristic absorption band centered at 556 nm (ε = 43300 M^{-1} cm^{-1}) and a weak fluorescence emission peak at 574 nm. The fluorescence quantum yield (Φ) was as low as 0.005, which is comparable with that of RosAg, indicating that the hexathioether ligands did not interfere with the efficient PET quenching from the receptor to the xanthene moiety. Upon addition of Hg^{2+}, the fluorescence increased by 20-fold (Φ = 0.11) with slight bathochromic shifts in the excitation (557 nm; ε = 48600 M^{-1} cm^{-1}) and emission (579 nm) maxima (Figure S3 in the SI). The fluorescence intensity at 579 nm linearly increased up to a 1:1 $[Hg^{2+}]/[RosHg]$ ratio (Figure S4 in the SI), which is consistent with a 1:1 complex stoichiometry. The formation of a 1:1 complex was further confirmed by Job's plot, where the intensity at 579 nm reached the maximum when the molar fraction of Hg^{2+} was 0.5 (Figure S5 in the SI). In order to determine a reliable dissociation constant K_d for Hg^{2+} and the RosHg complex, we used a set of Hg^{2+}/Mg^{2+}/ethylenediaminetetraacetic acid (EDTA) buffer solutions, which provided various concentrations of $[Hg^{2+}]_{free}$ ranging from 2×10^{-18} to 1×10^{-15} M (Figure 1a). On the basis of nonlinear curve-fitting analysis of

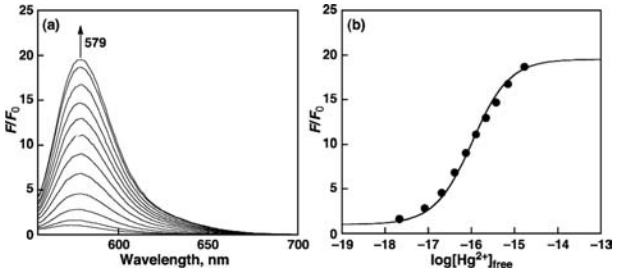

Figure 1. (a) Emission spectra of RosHg (2 μM) with an excitation wavelength at 550 nm as a function of the concentration of free Hg^{2+} in a mixture of HEPES buffer (pH 7.20, 0.1 M KNO_3) and DMSO (4:1, v/v) containing 1 mM EDTA, 1 mM $MgSO_4$, and 0−0.9 mM $HgCl_2$. For the final spectrum, $HgCl_2$ was added to the solution of 2 μM RosHg in a mixture of HEPES buffer and DMSO (4:1, v/v). (b) Plots of the observed fluorescence intensities at 579 nm with best-fit curves for the dissociation constant 1.04×10^{-16} M.

the emission intensities (F/F_0) at each $[Hg^{2+}]_{free}$, K_d was calculated to be $1.04 \pm 0.05 \times 10^{-16}$ M (Figure 1b), which is much lower than the allowable level for Hg^{2+} in drinking water suggested by the U.S. Environmental Protection Agency, 2 ppb (10 nM). To the best of our knowledge, the high Hg^{2+} binding affinity of RosHg makes it the strongest of the reversible fluorescent Hg^{2+} sensors reported to date.

We next confirmed the fluorescence reversibility of RosHg upon Hg^{2+} binding. Glutathione (GSH: reduced form of γ-L-glutamyl-L-cysteinylglycine) is the most abundant cellular thiol compound, ranging from 0.5 to 10 mM in cells, and plays a pivotal role in heavy-metal detoxification.[16] Because of the extremely high stability constant (β) for the $Hg(GS)_2$ complex (log β = 40.95 at physiological pH),[17] it is predicted that an excess amount of GSH will deprive bound Hg^{2+} from the Hg^{2+}-RosHg complex, resulting in reproduction of the nonfluorescent metal-free form. As expected, the addition of 2 mM GSH to the aqueous solution of the Hg^{2+}-RosHg complex resulted in an immediate decrease in the fluorescence intensity to the original value of the metal-unbound RosHg (Figure S6 in the SI). This reversible binding feature of RosHg and Hg^{2+} implies that this sensor may be a useful chemical tool to monitor not only the cellular uptake of Hg^{2+} but also the increase of the intracellular GSH concentration, which induces the increase of mercury elimination from cells and tissues.

The selectivity profiles of RosHg were examined by titration with various metal ions (Figure 2). High concentrations (5 mM)

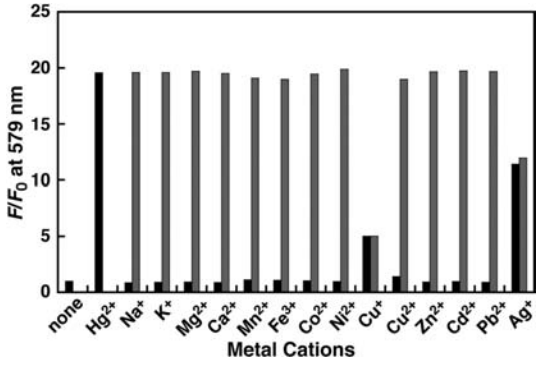

Figure 2. Metal-ion selectivity of RosHg in response to various metal ions in a mixture of HEPES buffer (50 mM, pH 7.20, 0.1 M KNO_3) and DMSO (4:1, v/v). Bars represent the emission response (F) over the initial emission intensity (F_0) at 579 nm. Black bars represent the addition of heavy-metal ion (5 μM) or Na^{+}, K^{+}, Ca^{2+}, and Mg^{2+} (5 mM) to a probe solution (2 μM). Gray bars represent the measurements subsequent to the addition of 5 μM Hg^{2+} to the solution. The counterions used for all metal ions were chloride, except for Cu^{+}, which was prepared from $[Cu(CH_3CN)_4]PF_6$.

of alkali- and alkali-earth-metal ions, which are highly abundant species in drinking and natural water and living samples, induced little change in the emission spectra of RosHg. This exerted a negligible effect on the fluorescence response for Hg^{2+}, indicating the potential use of this sensor for biological samples. RosHg was selective for Hg^{2+} over first-row transition metals including Mn^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+}, and Zn^{2+}, as well as the heavy-metal ions Cd^{2+} and Pb^{2+}, both commonly associated with environmental pollutants. Because the multithioether units are also a possible ligand for Cu^{+} and Ag^{+}, these metal ions induced relatively smaller fluorescence enhancement of RosHg and interfered with the response to Hg^{2+}.

For practical application of RosHg, fluorescence imaging for Hg^{2+} was examined in living cells using confocal fluorescence microscopy. HeLa cells incubated with 1 μM RosHg showed very weak but detectable fluorescence inside the cells. This staining pattern correlated well with mitochondrial staining with MitoTracker Green FM, a mitochondrial indicator, indicating that RosHg is localized selectively at mitochondria (Figure S7 in the SI). The cells were then exposed to 5 μM Hg^{2+} for 30 min at 37 °C, and the fluorescence images were taken after washing with phosphate-buffered saline containing 2 mM EDTA to remove extracellular Hg^{2+} (Figure S8 in the SI). However, little increase

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in fluorescence was observed, suggesting that intracellular thiol compounds such as GSH and metallothionein form extremely stable Hg^{2+} complexes and, hence, play a role in heavy-metal detoxification. This result is consistent with the in vitro observation that RosHg exhibits no fluorescence enhancement to Hg^{2+} in the presence of 2 mM GSH. We subsequently used HeLa cells pretreated with 1 mM *N*-ethylmaleimide (NEM), which is a membrane-permeable sulfhydryl blocker,[18] for 30 min at 37 °C before imaging to decrease the intracellular GSH level. In contrast to cells not treated with NEM, strong fluorescence was seen in cells stained with more diluted RosHg (the final concentration was 200 nM) after exposure to 5 μM Hg^{2+} for 30 min (Figure 3a,b). Again, costaining experiments with

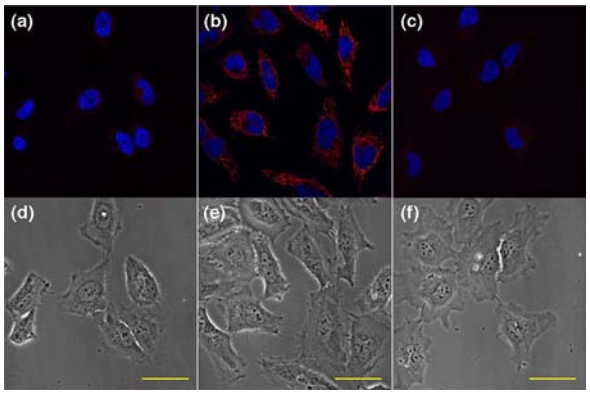

Figure 3. Confocal fluorescence images of HeLa cells pretreated with 1 mM NEM for 30 min at 37 °C: (a) Fluorescence image of cells incubated with 200 nM RosHg for 30 min at 37 °C. (b) Cells in panel a exposed to 5 μM $HgCl_2$ for 30 min at 37 °C. (c) Same cells then treated with 1 mM NAC and incubated for 1 h at 37 °C. (d–f) Bright fields of each image. Nuclei (blue) of cells were visualized by Hoechst 33258 staining. Scale bar = 50 μm.

MitoTracker Green FM demonstrated that RosHg was retained in the mitochondria and Hg^{2+} was detected locally in the cells (Figure S9 in the SI).

In order to confirm whether RosHg can exhibit a reversible fluorescence response toward Hg^{2+} in living cells, *N*-acetylcysteine (NAC), which is rapidly metabolized to intracellular GSH,[19] was added to the same culture media (the final concentration of NAC was 1 mM). After incubation for 1 h at 37 °C, the observed fluorescence increase was reversed to background levels (Figures 3c and S10 in the SI). These imaging results demonstrate that RosHg is suitable to visualize the changes of intracellular unbound-Hg^{2+} concentrations and can directly monitor the Hg^{2+}-GSH biological detoxification system conjugations at the cellular level.

In summary, we have reported the fluorescence sensor RosHg for reversible detection of Hg^{2+} with femtomolar binding affinity in an aqueous solution. RosHg was successfully used for monitoring the changes of the Hg^{2+} level in the mitochondria of living cells. Studies of the intracellular distribution of inorganic mercury have demonstrated that mitochondria are the major target organelle for mercury in cells of the liver and kidney.[20] This probe is expected to be a useful tool for investigations not only of the metabolic pathway and toxicity of a mercury ion in organs but also of glutathione and metallothionein biosynthesis for mercury detoxification.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Detailed descriptions of the synthetic procedure and characterizations of new compounds, ^1H NMR and UV–vis absorption spectra of the Hg^{2+}-RosHg complex, plots of Hg^{2+} titration, Job's plot, and fluorescence images of cells with RosHg. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interests.

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