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In Vivo Monitoring of Mercury Ions Using a Rhodamine-Based Molecular Probe

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Abstract: Exposure to mercury causes severe damage to various tissues and organs in humans. Concern over mercury toxicity has encouraged the development of efficient, sensitive, and selective methods for the in vivo detection of mercury. Although a variety of chemosensors have been exploited for this purpose, no in vivo monitoring systems have been described to date. In this report, we describe an irreversible rhodamine chemosensor-based, real-time monitoring system to detect mercury ions in living cells and, in particular, vertebrate organisms. The chemosensor responds rapidly, irreversibly, and stoichiometrically to mercury ions in aqueous media at room temperature. The results of experiments with mammalian cells and zebrafish show that the mercury chemosensor is cell and organism permeable and that it responds selectively to mercury ions over other metal ions. In addition, real-time monitoring of mercury-ion uptake by cells and zebrafish using this chemosensor shows that saturation of mercury-ion uptake occurs within 20−30 min in cells and organisms. Finally, accumulation of mercury ions in zebrafish tissue and organs is readily detected by using this rhodamine-based chemosensor.

Introduction

Mercury, one of the most prevalent toxic metals in the environment, easily passes through biological membranes such as skin, respiratory, and gastrointestinal tissues.[1] When absorbed in the human body, mercury causes damage to the central nervous and endocrine system.[2] Long-term exposure to high levels of this metal leads to permanent deterioration of the brain, kidneys, and developing fetus. In particular, brain damage induced by mercury causes several symptoms including irritability, tremors, and vision, hearing, and memory impairment. Short-term exposure to high levels of mercury results in lung damage, nausea, vomiting, and diarrhea. Furthermore, organisms exposed to inorganic mercury develop autoimmune disease caused by inhibition of apoptotic activities required for T-cell homeostasis.[3]

Concern over the toxicity of mercury has stimulated explorations aimed at developing selective and efficient methods to monitor mercury in cells and, in particular, organisms. Highly selective and sensitive fluorescent chemosensors have been extensively investigated for the purpose of in vitro detection of specific metal ions and in vivo applications for analyzing biologically important metal ions in cells.[4−8] Metal-selective fluorescent chemosensors have been developed for assessing biologically relevant metal ions, such as calcium(II),[5] zinc(II),[6] copper(I),[7] and magnesium(II)[8] in cells. Although various fluorescent[9] and colorimetric[10] chemosensors for mercury ions have been applied to in vitro detection, in vivo monitoring systems for this deleterious metal ion have not yet been developed.[11]

Recently, we uncovered a rhodamine-based fluorescent chemosensor **1** which can be used as a selective probe for mercury ions in aqueous solution.[12] Unlike other chemosensors that respond to mercury in a reversible manner, this system reacts irreversibly with mercury ions to produce **2** (Figure 1), a

(1) Gutknecht, J. *J. Membr. Biol.* **1981**, *61*, 61−66.
(2) (a) Clarkson, T. W.; Magos, L.; Myers, G. J. *N. Engl. J. Med.* **2003**, *349*, 1731−1737. (b) Von Burg, R. *J. Appl. Toxicol.* **1995**, *15*, 483−493.
(3) (a) Ziemba, S. E.; McCabe, M. J., Jr.; Rosenspire, A. J. *Toxicol. Appl. Pharmacol.* **2005**, *206*, 334−342. (b) Guo, T. L.; Miller, M. A.; Shapiro, I. M.; Shenker, B. J. *Toxicol. Appl. Pharmacol.* **1998**, *153*, 250−257.
(4) (a) In *Molecular Fluorescence: Principles and Applications*; Valeur, B., Ed.; Wiley-VCH: Weinheim, 2002. (b) In *Fluorescent Chemosensors for Ion and Molecular Recognition*; Czarnik, A. W., Ed.; ACS Symposium Series 538; American Chemical Society: Washington, DC, 1993. (c) de Silva, A. P.; Gunaratne, H. Q. N.; Gunnlaugsson, T. A.; Huxley,. J. M.; McCoy, C. P.; Rademacher, J. T.; Rice, T. E. *Chem. Rev.* **1997**, *97*, 1515−1566. (d) Fabbrizzi, L.; Poggi, A. *Chem. Soc. Rev.* **1995**, 197−202. (e) Czarnik, A. W. *Acc. Chem.* Res. **1994**, *27*, 302−308.
(5) (a) Komatsu, H.; Miki, T.; Citterio, D.; Kubota, T.; Shindo, Y.; Kitamura, Y.; Oka, K.; Suzuki, K. *J. Am. Chem. Soc.* **2005**, *127*, 10798−10799. (b) Tsien, R. W.; Tsien, R. Y. *Annu. Rev. Cell Biol.* **1990**, *6*, 715−760.
(6) (a) Bozym, R. A.; Thompson, R. B.; Stoddart, A. K.; Fierke, C. A. *ACS Chem. Biol.* **2006**, *1*, 103−111. (b) Füssl, A.; Schleifenbaum, A.; Göritz, M.; Riddell, A.; Schultz, C.; Krämer, R. *J. Am. Chem. Soc.* **2006**, *128*, 5986−5987. (c) Leevy, W. M.; Johnson, J. R.; Lakshmi, C.; Morris, J.; Marquez, M.; Smith, B. D. *Chem. Commun*. **2006**, 1595−1597. (d) Nolan, E. M.; Jaworski, J.; Okamoto, K.-I.; Hayashi, Y.; Sheng, M.; Lippard, S. J. *J. Am. Chem. Soc.* **2005**, *127*, 16812−16823. (e) Lim, N. C.; Freake, H. C.; Brückner, C. *Chem.*—Eur. J. **2005**, *11*, 38−49. (f) Kikuchi, K.; Komatsu, K.; Nagano, T. *Curr. Opin. Chem. Biol.* **2004**, *8*, 182−191. (g) Taki, M.; Wolford, J. L.; O'Halloran, T. V. *J. Am. Chem. Soc.* **2004**, *126*, 712−713. (h) Burdette, S. C.; Lippard, S. J. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 3605−3610. (i) Maruyama, S.; Kikuchi, K.; Hirano, T.; Urano, Y.; Nagano, T. *J. Am. Chem. Soc.* **2002**, *124*, 10650−10651. (j) Gee, K. R.; Zhou, Z.-L.; Qian, W.-J.; Kennedy, R. *J. Am. Chem. Soc.* **2002**, *124*, 776−778. (k) Walkup, G. K.; Burdette, S. C.; Lippard, S. L.; Tsien, R. Y. *J. Am. Chem. Soc.* **2000**, *122*, 5644−5645.
(7) Zeng, L.; Miller, E. W.; Pralle, A.; Isacoff, E. Y.; Chang, C. J. *J. Am. Chem. Soc.* **2006**, *128*, 10−11.
(8) (a) Farruggia, G.; Iotti, S.; Prodi, L.; Montalti, M.; Zaccheroni, N.; Savage, P. B.; Trapani, V.; Sale, P.; Wolf, F. I. *J. Am. Chem. Soc.* **2006**, *128*, 344−350. (b) Komatsu, H.; Iwasawa, N.; Citterio, D.; Suzuki, Y.; Kubota, T.; Tokuno, K.; Kitamura, Y.; Oka, K.; Suzuki, K. *J. Am. Chem. Soc.* **2004**, *126*, 16353−16360.

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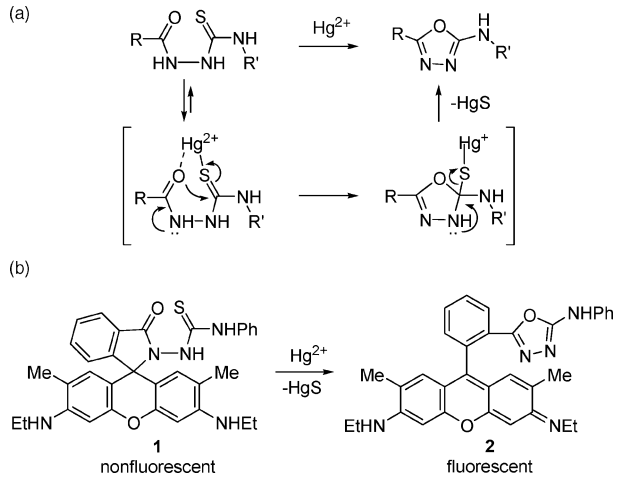

Figure 1. (a) Hg^{2+}-promoted cyclization of thiosemicarbazide to 1,3,4-oxadiazole. (b) Conversion of nonfluorescent **1** to strongly fluorescent **2** by mercury ions.

substance that is strongly fluorescent at long wavelengths (excitation at 500 nm, emission at 557 nm) where background autofluorescence in cells is minimal. Since rhodamine-based chemosensors have been widely used for detecting intracellular analytes,[13] we expected that **1** would be applicable for monitoring mercury ions in living cells and organisms. Below, we describe the results of an investigation that demonstrates that **1** serves as the foundation for an irreversible chemosensor-based, real-time method for monitoring mercury ions in living cells and, in particular, vertebrate organisms.

Results and Discussion

Chemical and Spectroscopic Properties of 1. Unlike conventional chemosensors that operate by reversible binding to metal ions, chemodosimeters that utilize an irreversible chemical event are of greater utility for ratiometric determination of analyte concentrations due to their potentially high sensitivity and selectivity. Although many thiophilic mercury-selective chemodosimeters have been developed thus far,[14] they have several disadvantages in the context of the criteria required for in vivo applications. For example, desulfurization reactions, which drive mercury-ion-induced fluorescent changes in these systems, often require either elevated temperatures or excess amounts of Hg^{2+} for completion.[15] In addition, in vivo applications require that the chemodosimeters operate in aqueous media and respond to low levels of mercury ions. Finally, since the desulfurization reaction of the chemodosimeters can be also promoted by other metal ions, such as Ag^+ and Pb^{2+}, selectivity is an important issue.

Chemosensor **1** has ideal chemical and spectroscopic properties that satisfy the criteria for in vivo applications.[12] The thiosemicarbazide group in this substance undergoes irreversible mercury-ion-promoted cyclization to form a 1,3,4-oxadiazole group in **2** in aqueous media at room temperature (Figure 1). Although the mercury-ion-promoted oxadiazole-forming process has been reported to take place at slightly elevated temperatures,[16] this process in **1** occurs at room temperature, a likely consequence of the electron-donating ability of xanthene ring nitrogens. Also, the results of a pH titration experiment revealed that **1** is stable over a wide pH range (4−14).

Upon addition of mercury ions, a solution of **1** in PBS buffer (pH 7.4) instantaneously changes from colorless to pink. In addition, the solution becomes strongly fluorescent at long wavelength (emission maximum at 557 nm). The substance produced in this process, **2**, has a large molar absorptivity (log $\epsilon = 4.67$) and a high fluorescence quantum yield ($\Phi_f = 0.52$).[17] Therefore, the fluorescent spectroscopic change that accompanies conversion of **1** to **2** was observed at ppb levels in PBS buffer.[18] Fluorescence monitoring of a titration of Hg^{2+} (0.05−2.0 equiv) using 1 μM **1** in PBS (pH 7.4) showed that the fluorescence intensity at 557 nm is directly proportional to the amount of Hg^{2+} and the increase of fluorescence intensity stops after 1 equiv of Hg^{2+} (Figure 2a). This behavior demonstrates that chemodosimeter **1** responds to Hg^{2+} in 1:1 stoichiometry. Support for this conclusion was gained by comparing the fluorescence intensity profiles obtained from reactions of **1** and Hg^{2+} (1:1 ratio, 0.1−12 μM in PBS, pH 7.4) with that obtained from the corresponding concentrations of **2** (0.1−12 μM) (Figure 2b). Another important observation is that **1** is unreactive in the presence of other metal ions, such as Ag^+, Zn^{2+}, Cu^{2+}, Pb^{2+},

(9) Examples of Hg^{2+}-selective fluorescent chemosensors: (a) Zhu, X.-J.; Fu, S.-T.; Wong, W.-K.; Guo, J.-P.; Wong, W.-Y. *Angew. Chem., Int. Ed.* **2006**, *45*, 3150−3154. (b) Zheng, H.; Qian, Z.-H.; Xu, L.; Yuan, F.-F.; Lan, L.-D.; Xu, J.-G. *Org. Lett.* **2006**, *8*, 859−861. (c) Kim, S. H.; Kim, J. S.; Park, S. M.; Chang, S.-K. *Org. Lett.* **2006**, *8*, 371−374. (d) Mello, J. V.; Finney, N. S. *J. Am. Chem. Soc.* **2005**, *127*, 10124−10125. (e) Moon, S.-Y.; Youn, N. J.; Park, S. M.; Chang, S.-K. *J. Org. Chem.*. **2005**, *70*, 2394−2397. (f) Chen, Q.-Y.; Chen, C.-F. *Tetrahedron Lett.*. **2005**, *46*, 165−168. (g) Guo, X. G.; Qian, X.; Jia, L. *J. Am. Chem.* Soc. **2004**, *126*, 2272−2273. (h) Kwon, J. Y.; Soh, J. H.; Yoon, Y. J.; Yoon, J. *Supramol. Chem.* **2004**, *16*, 621−624. (i) Ono, A.; Togashi, H. *Angew. Chem., Int. Ed.* **2004**, *43*, 4300−4302. (j) Ros-Lis, J. V.; Martínez-Máñez, R.; Rurack, K.; Sancenón, F.; Soto, J.; Spieles, M. *Inorg. Chem.* **2004**, *43*, 5183−5185. (k) Nolan, E. M.; Lippard, S. J. *J. Am. Chem. Soc.* **2003**, *125*, 14270−14271. (l) Descalzo, A. B.; Martínez-Máñez, R.; Radeglia, R.; Rurack, K.; Soto, J. *J. Am. Chem. Soc.* **2003**, *125*, 3418−3419. (m) Choi, M. J.; Kim, M. Y.; Chang, S.-K. *Chem. Commun.* **2001**, 1664−1665. (n) Prodi, L.; Bargossi, C.; Montalti, M.; Zaccheroni, N.; Su, N.; Bradshaw, J. S.; Izatt, R. M.; Savage, P. B. *J. Am. Chem. Soc.* **2000**, *122*, 6769−6770. (o) Rurack, K.; Kollmannsberger, M.; Resch-Genger, U.; Daub, J. *J. Am. Chem. Soc.* **2000**, *122*, 968−969. (p) Sakamoto, H.; Ishikawa, J.; Nakao, S.; Wada, H. *Chem. Commun.* **2000**, 2395−2396.

(10) (a) Caballero, A.; Martínez, R.; Lioveras, V.; Tatera, I.; Vidal-Gancedo, J.; Wurst, K.; Tárraga, A.; Molina, P.; Veciana, J. *J. Am. Chem. Soc.* **2005**, *127*, 15666−15667. (b) Ros-Lis, J.; Marcos, M. D.; Martínez-Máñez, R.; Soto, J. *Angew. Chem., Int. Ed.* **2005**, *44*, 4405−4407. (c) Coronado, E.; Galán-Mascarós, J. R.; Martí-Gastaldo, C.; Palomares, E.; Durrant, J. R.; Vilar, R.; Gratzel, M.; Nazeeruddin, Md. K. *J. Am. Chem. Soc.* **2005**, *127*, 12351−12356. (d) Huang, J.-H.; Wen, W.-H.; Sun, Y.-Y.; Chou, P.-T.; Fang, J.-M. *J. Org. Chem.*. **2005**, *70*, 5827−5832. (e) Palomares, E.; Vilar, R.; Durrant, J. R. *Chem. Commun.* **2004**, 362−363. (f) Sancenón, F.; Martínez-Máñez, R.; Soto, J. *Tetrahedron Lett.* **2004**, *42*, 4321−4323. (g) Brümmer, O.; La Clair, J. J.; Janda, K. D. *Org. Lett.* **1999**, *1*, 415−418.

(11) For quantitative analysis of mercury ions in fish using a fluorescence chemosensor, see: Yoon, S.; Albers, A. E.; Wong, A. P.; Chang, C. J. *J. Am. Chem. Soc.* **2005**, *127*, 16030−16031.

(12) Yang, Y.-K.; Yook, K.-J.; Tae, J. *J. Am. Chem. Soc.* **2005**, *127*, 16760−16761.

(13) In *The Handbook-A Guide to Fluorescent Probes and Labeling Technologies*, 10th ed.; Haugland, R. P., Ed.; Molecular Probes: Eugene, OR, 2005.

(14) (a) Chae, M.-Y.; Czarnik, A. W. *J. Am. Chem. Soc.* **1992**, *114*, 9704−9705. (b) Dujols, V.; Ford, F.; Czarnik, A. W. *J. Am. Chem. Soc.* **1997**, *119*, 7386−7387. (c) Hennrich, G.; Sonnenschein, H.; Resch-Genger, U. *J. Am. Chem. Soc.* **1999**, *121*, 5073−5074. (d) Zhang, G.; Zhang, D.; Yin, S.; Yang, X.; Shuai, Z.; Zhu, D. *Chem. Commun.* **2005**, 2161−2163. (e) Liu, B.; Tian, H. *Chem. Commun.* **2005**, 3156−3158. (f) Ros-Lis, J.; Marcos, M. D.; Martínez-Máñez, R.; Soto, J. *Angew. Chem., Int. Ed.* **2005**, *44*, 4405−4407. (g) Zheng, H.; Qian, Z.-H.; Xu, L.; Yuan, F.-F.; Lan, L.-D.; Xu, J.-G. *Org. Lett.* **2006**, *8*, 859−861. (h) Song, K. C.; Kim, J. S.; Park, S. M.; Chung, K.-C.; Ahn, S.; Chang, S.-K. *Org. Lett.* **2006**, *8*, 3413−3416.

(15) Typical desulfurization reactions used in known Hg^{2+} chemodosimeters require elevated temperatures and/or relatively long reaction times. See ref 14.

(16) (a) Wang, X.; Li, Z.; Wei, B.; Yang, J. *Synth. Commun.* **2002**, *32*, 1097−1103. (b) Zou, X.; Jin, G. *J. Heterocycl. Chem.* **2001**, *38*, 993−996.

(17) The fluorescence quantum yield was calculated by using Rhodamine 6G ($\Phi_f = 0.94$ in EtOH) as a reference (Fischer, M.; Georges, J. *Chem. Phys. Lett.* **1996**, *260*, 115−116).

(18) The EPA standard for the maximum allowable amount of Hg^{2+} in drinking water is 2 ppb.

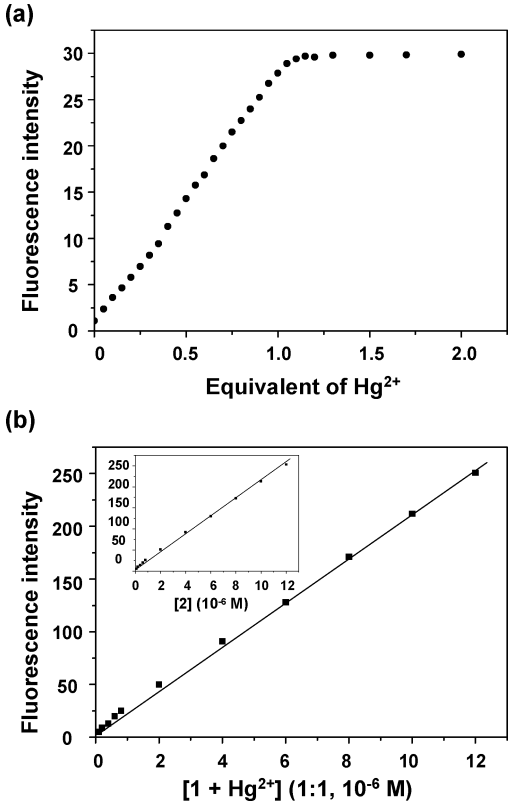

Figure 2. Stoichiometric response of **1** to mercury ions. (a) Fluorescence response of **1** (1 μM) to $HgCl_2$ (0.05−2.0 equiv) in PBS (pH 7.4) (excitation at 500 nm, emission at 557 nm). (b) Fluorescence intensity profile obtained from reactions of **1** and Hg^{2+} (1:1 ratio, 0.1−12 μM, in PBS, pH 7.4). The inset is a graph of fluorescence intensity of **2** as a function of concentration (0.1−12 μM) in PBS (pH 7.4). Fluorescence intensity was measured by a spectrofluorimeter.

Cd^{2+}, Ni^{2+}, Co^{2+}, Fe^{2+}, Mn^{2+}, Ca^{2+}, Ba^{2+}, and Cr^{2+}, in aqueous solutions, indicating that this chemodosimeter displays a high Hg^{2+} selectivity (see Figure S1 in Supporting Information).[19]

Kinetics of the Response of 1 to Mercury Ions. The time dependence of the response of **1** to mercury ions in aqueous solution was determined. Kinetic studies were performed using a stopped-flow spectrophotometer at 25 °C under pseudo-first-order reaction conditions (2.5 μM **1** and 2.5 mM $HgCl_2$ in PBS).[20] The time-dependent response of **1** to mercury ions was monitored using absorption spectroscopy (Figure 3). The observed rate constant (k_{obs}) was found to be 3.5 ± 0.17 s^{-1} ($t_{1/2}$ = 0.2 s), indicating that **1** is rapidly converted to **2** under these conditions. We also investigated the time-dependent fluorescence response of **1** (10 μM) in the presence of 1 equiv of Hg^{2+} (10 μM) in PBS. It was revealed that the reaction was completed in less than 1 min (see Figure S1b in Supporting Information). Thus, this system could be used for real-time monitoring of mercury ions in cells and organisms.

In Vivo Detection of Mercury Ions. Owing to its chemical and spectroscopic properties, **1** should be ideally suited to monitoring mercury ions in living cell and organisms. To test this proposal, in vivo detection of mercury ions in mouse muscle precursor cells and rat neuron cells was evaluated. In addition, human muscle precursor cells were also used for in vivo detection of mercury ions. Murine C2C12 myoblasts were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS). Human skeletal muscle precursor cells were obtained by incubating single muscle fibers isolated from human muscle sample in DMEM containing 10% FBS for 5−6 days.[21] The rat neuron cells were obtained by differentiating rat PC12 cells using 10−100 ng/mL of nerve growth factor for 7 days.[22] The cultured muscle cells and differentiated neuron cells were exposed to 50 μM $HgCl_2$ for 10−20 min at 37 °C, washed with PBS to remove the remaining mercury ions, and incubated with 50 μM **1** for 20 min. In a reverse fashion, the muscle and neuron cells were first treated with 50 μM **1** for 20 min and then incubated with 50 μM $HgCl_2$ for 10 min at 37 °C after removal of the remaining chemosensor. Fluorescence microscopic images of the cells subjected to the two procedures show that **1** enters cell membranes and reacts with mercury ions to form the fluorescent product **2** (Figure 4a−c).[23] In addition, the detection limit of mercury ions in the in vivo system was also examined by measuring the fluorescence intensity of the treated C2C12 cells with a fluorescence microplate reader. It was found that mercury ions in C2C12 cells treated with more than 40 μM Hg^{2+} were monitored by this method (see Figure S4 in Supporting Information).

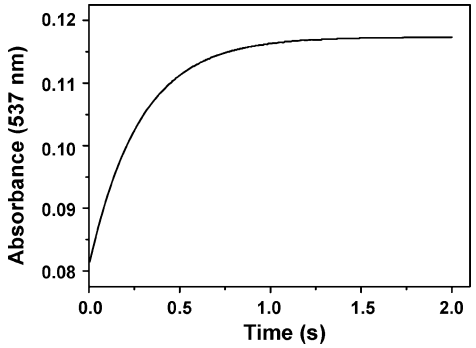

Figure 3. Time course of the response of **1** to Hg^{2+}. Kinetic studies were performed using a stopped-flow spectrophotometer at 25 °C under pseudo-first-order conditions (2.5 μM **1** and 2.5 mM $HgCl_2$ in PBS, pH 7.4). The increase of absorbance at 537 nm produced by reaction of **1** and mercury ions was continuously monitored at time intervals.

The success encountered in the cell experiments encouraged an exploratory effort to determine if chemosensor **1** could be used to detect mercury ions in living organisms. Five-day-old zebrafish was treated with 50 μM $HgCl_2$ in E3 embryo media for 10 min at 28 °C,[24] washed with PBS to remove the remaining mercury ions, and incubated in a solution containing 50 μM **1** for 20 min. In the reverse fashion, the zebrafish was first incubated with 50 μM **1** for 20 min at 28 °C and then exposed

(19) The fluorescence response of **1** to other biologically relevant mercury species such as CH_3HgCl in PBS was also investigated. It was revealed that the response of **1** to CH_3HgCl is slower and less effective than $HgCl_2$. Fluorescence intensity changes of **1** in the presence of 1 equiv of CH_3HgCl were undetectable unlike $HgCl_2$. However, excess amounts of CH_3HgCl induced the increase of fluorescence intensity (see Figure S2b in Supporting Information). The reaction product of **1** and CH_3HgCl was identified to be **2** by NMR analysis.

(20) Jencks, W. P. *Catalysis in Chemistry and Enzymology*; Dover Publications, Inc., Mineola, NY, 1987; pp 568−590.

(21) Bonavaud, S.; Agbulut, O.; D'Honneur, G.; Nizard, R.; Mouly, V.; Butler-Browne, G. *In Vitro Cell Dev. Biol. Anim.* **2002**, *38*, 66−72.

(22) Tischler, A. S.; Greene, L. A. *Proc. Natl. Acad. Sci. U.S.A.* **1976**, *73*, 2424−2428.

(23) The compound generated from **1** in the presence of mercury ions in cells was confirmed to be **2** by comparison of the retention time of HPLC and mass spectrum of the compound with those of reference compound **2** (see Figure S3 in Supporting Information).

(24) (a) Westerfield, M. *The Zebrafish Book*, 3rd ed.; University of Oregon Press: Eugene. OR, 2000. (b) Nusslein-Volhard, C.; Dahm, R. *Zebrafish. A Practical Approach*; Oxford University Press: Germany, 2002.

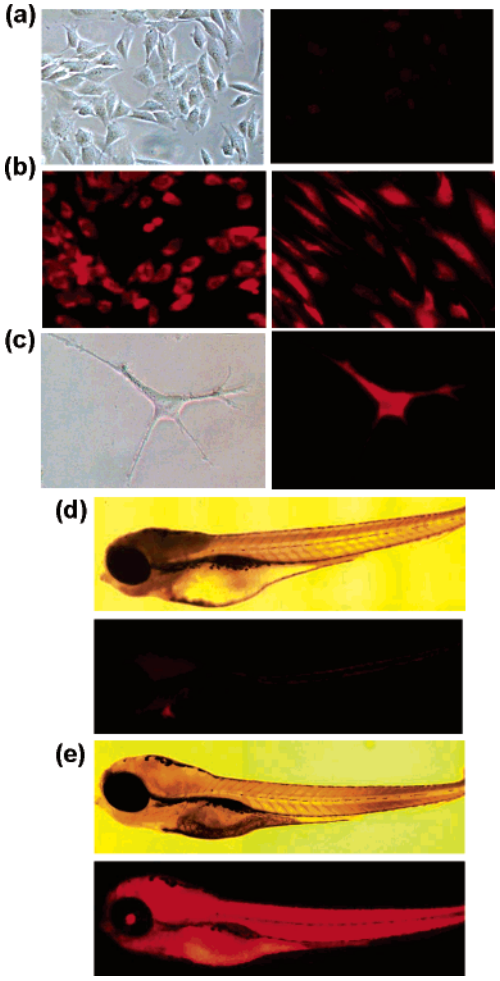

Figure 4. Cells and organisms incubated with **1** (50 μM) and $HgCl_2$ (50 μM). The mammalian cells and 5-day-old zebrafish were treated with **1** for 20 min, washed with PBS to remove the remaining chemosensor, and incubated with $HgCl_2$ for 10 min. (a) Images of C2C12 cells treated with **1** in the absence of external mercury ions (left, microscopic image; right, fluorescence microscopic image), (b) fluorescence images of murine C2C12 myoblasts (left) and human muscle precursor cells (right) treated with both **1** and mercury ions, (c) images of neurons differentiated from rat PC12 cells treated with both **1** and mercury ions (left, microscopic image; right, fluorescence microscopic image), (d) images of 5-day-old zebrafish treated with **1** in the absence of external mercury ions (top, microscopic image; bottom, fluorescence microscopic image), and (e) images of 5-day-old zebrafish treated with both **1** and mercury ions (top, microscopic image; bottom, fluorescence microscopic image).

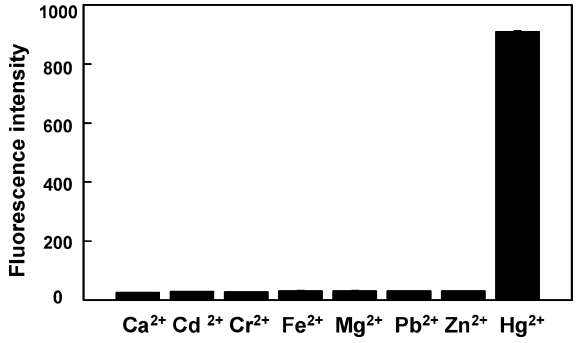

Figure 5. Fluorescence response of **1** (10 μM) to various metal ions (200 μM) in C2C12 cells. Fluorescence intensity was measured by a fluorescence microplate reader.

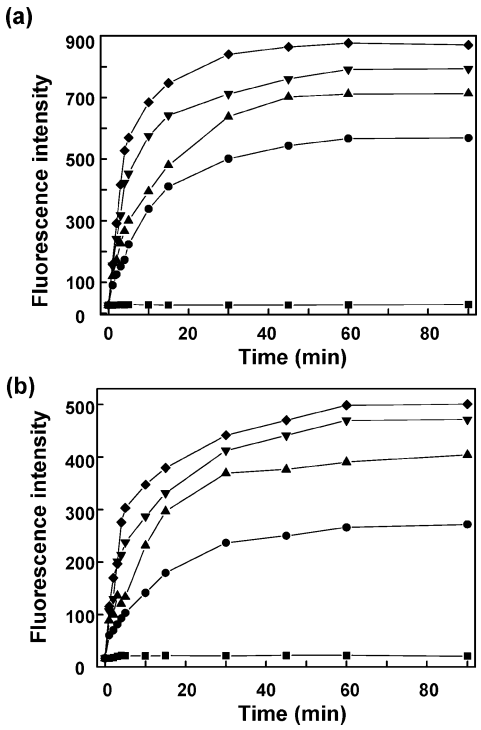

Figure 6. Real-time monitoring of Hg^{2+} uptake by (a) C2C12 cells and (b) 5-day-old zebrafish using 200 μM **1** ($HgCl_2$; (■) 0, (●) 40, (▲) 60, (▼) 80, and (◆) 100 μM). The change of fluorescence intensity of cells and organisms was continuously monitored by a fluorescence microplate reader (excitation at 500 nm, emission at 557 nm).

to 50 μM $HgCl_2$ for 10 min after removal of the remaining chemosensor. The results of fluorescence microscope analysis of these specimens show that mercury ions in zebrafish are fluorescently detected by **1** (Figure 4d and e).

The ability of chemosensors to selectively monitor metal ions in cells is an important requirement for biological applications. As described above, chemosensor **1** promotes a mercury-ion-selective fluorescence response in vitro. To examine whether this specificity is preserved in in vivo systems, mammalian C2C12 cells were treated with 10 μM **1** for 20 min, washed with PBS to remove the remaining chemosensor, and individually exposed to 200 μM of various metal ions including Mg^{2+}, Zn^{2+}, Ca^{2+}, Fe^{2+}, Cd^{2+}, Mn^{2+}, Pb^{2+}, and Hg^{2+} for 20 min. The fluorescence analysis of these cells reveals that chemosensor **1** exhibits a greater than 50-fold selective response for mercury ions over other biologically relevant ions, such as Mg^{2+}, Zn^{2+}, Ca^{2+}, and Fe^{2+} (Figure 5). Importantly, **1** shows a 50-fold greater response to mercury ions than to Cd^{2+} and Pb^{2+}, prevalent toxic metals in the environment.

Real-Time Monitoring of Mercury-Ion Uptake in Cells and Organisms. Owing to its cell permeability and rapid fluorescence response to Hg^{2+}, **1** should be applicable to the continuous monitoring of mercury-ion uptake by cells and organisms. To test this proposal, time-dependent uptake of mercury ions was determined by incubating chemosensor-containing cells and organisms with mercury ions while measuring the increase of fluorescence intensity as a function of time. The C2C12 cells and 5-day-old zebrafish were incubated with 200 μM **1** for 30 min (the cells and zebrafish remain alive), washed with PBS, and then treated with 0−100 μM $HgCl_2$ for 1.5 h. As the data in Figure 6 show, mercury ions enter the cells and organisms within 20−30 min as judged by the continuous increase of fluorescence intensity with time. It is notable that mercury-ion uptake by the cells and organisms

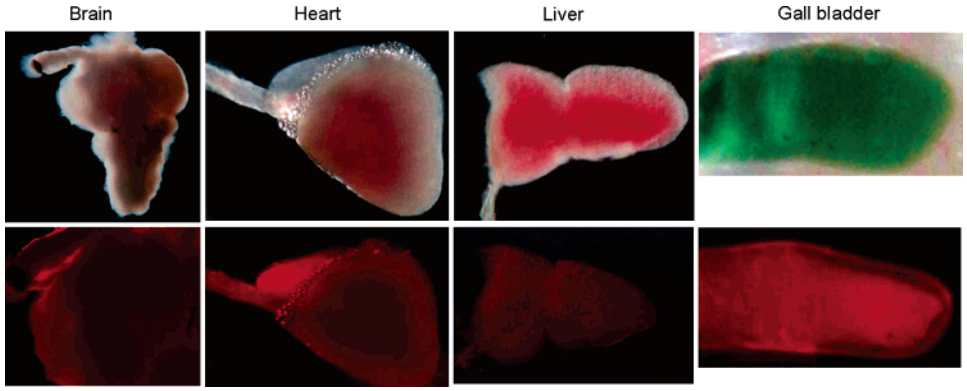

Figure 7. Images of zebrafish organs treated with 5 nM $HgCl_2$ and 10 μM **1** (top, microscopic images; bottom, fluorescence images). Also, see Supporting Information for images of zebrafish organs treated with only 10 μM **1** as a control.

shows saturation behavior and increases as the concentration of mercury ions increases even at a fixed concentration (200 μM) of **1**. This may imply that the cells and organisms absorb only a limited amount of mercury ions from the culture media at a level that depends on the external mercury-ion concentrations. The results of previous studies probing cellular uptake of mercury using radioactive ^{203}Hg also showed that mercury-ion uptake by cells reaches a maximum value within about 20 min.[25] However, it is difficult to perform continuous monitoring of mercury uptake by cells and organisms using the radioactive mercury-based methodology. In contrast, the technique that relies on the conversion of chemosensor **1** to fluorescent product **2** can be safely and conveniently employed for real-time monitoring of mercury-ion uptake by cells and organisms.

Detection of Mercury Ions Accumulated in Organisms. Experiments were designed to determine if **1** can be used to detect the accumulation of mercury ions in living organisms. Adult zebrafish (3 months old with identifiable organs) was exposed to 5 nM mercury ions in E3 media for 12 h, washed with PBS, and then incubated with 10 μM **1** for 30 min. In a separate experiment, zebrafish, after being exposed to 5 nM mercury ions in E3 media for 12 h, was transferred to mercury-free E3 media, grown for an additional 12 h, and then incubated with 10 μM **1** for 30 min. Zebrafish, treated under both conditions, were dissected to isolate tissues and organs that were then examined using fluorescence microscopy. Fluorescence intensities of isolated tissues and organs were analyzed using Image Pro Plus version 5.1 software. Similar levels of fluorescence intensities were observed at different locations in zebrafish irrespective of the incubation conditions used (Figure 7), indicating nonspecific accumulation of mercury ions in zebrafish under the experimental conditions. Interestingly, mercury ions were detected in the brain, heart, live, and gall bladder of the zebrafish, suggesting that **1** is able to reach all of these organs.

Conclusion

A new in vivo method to monitor mercury ions, using the highly selective and sensitive chemodosimeter **1**, has been developed. This chemodosimeter responds to mercury ions stoichiometrically, rapidly, and irreversibly at room temperature as a consequence of a chemical reaction that produces the strongly fluorescent cyclization product **2**. This system can be employed to detect mercury ions in living cells and, in particular, vertebrate organisms. Excellent sensitivity and selectivity for mercury ions over competing metal ions in cells is observed with this chemosensor. The value of this system was demonstrated by its use in monitoring the real-time uptake of mercury ions in cells and organisms. Furthermore, this probe can be applied to monitoring the accumulation of mercury ions in organisms. To the best of our knowledge, this is the first example of a chemosensor-based methodology for in vivo monitoring of mercury ions. The observations made in this effort should serve as the foundation for new chemosensor-based biological investigations.

Experimental Section

General Synthetic Methods. The pH was recorded using an HI-8014 instrument (HANNA). UV absorption spectra were obtained on a HP 8452 UV/VIS Spectrophotometer (Hewlett-Packard). Fluorescence emission spectra were obtained using a Hitachi F-4500 spectrofluorimeter linked to a Pentium PC running the SpectraCalc software package. A circulating H_2O/MeOH bath was used during all experiments to regulate the temperature at 25.0 $\pm$ 0.1 °C. Samples were contained in 10.0 nm path length quartz cuvettes (3.5 mL volume). Upon excitation at 500 nm, the emission spectra were integrated over the range 520−640 nm. All measurements were conducted at least in triplicate. Fluorescence images were obtained using Nikon Eclipse TE2000 fluorescence microscopy. Preparation of rhodamine sensor **1** and all the in vitro experiments were performed according to the published procedure.[12]

Imaging of Mouse C2C12 Myoblasts Incubated with Mercury Ions and 1. C2C12 cells (American Type Culture Collection, Manassas, VA) were cultured in culture media (DMEM supplemented with 10% FBS, 50 unit/mL of penicillin, and 50 μg/mL of streptomycin) at 37 °C in a humidified incubator, and culture media were replaced with fresh media every day. C2C12 cells were seeded in a 6-well plate at a density of 10^4 cells per well in culture media. After 24 h, the cells were incubated with 50 μM $HgCl_2$ in culture media for 10 min at 37 °C. After washing with PBS to remove the remaining mercury ions, the treated cells were incubated with 50 μM **1** in culture media for 20 min at 37 °C.

Alternatively, C2C12 cells were incubated with 50 μM **1** in culture media for 20 min. After washing with PBS to remove the remaining sensor, the cells were exposed to 50 μM $HgCl_2$ for 10 min at 37 °C. The cells incubated under both conditions were imaged by fluorescence microscopy (excitation wavelength, 500 nm; emission wavelength, 557 nm).

Imaging of Neuron Cells Derived from Rat PC12 Cells Incubated with Mercury Ions and 1. PC12 cells (American Type Culture Collection, Manassas, VA) were cultured in culture media (DMEM

(25) Aduayom, I.; Denizeau, F.; Jumarie, C. *Cell Biol. Toxicol.* **2005**, *21*, 163−179.

supplemented with 10% FBS, 50 unit/mL of penicillin, and 50 μg/mL of streptomycin) at 37 °C in a humidified incubator, and culture media were replaced with fresh media every day. PC12 cells were seeded in a 6-well plate at a density of 10^3 cells per well in culture media. After 24 h, the culture media were replaced with differentiation media (culture media containing 10−100 ng/mL of nerve growth factor (Sigma)), and the cells were incubated for 7 days to differentiate into neuron cells.[20] The differentiation media were changed every 2−3 days. The neuron cells differentiated from PC12 cells were treated with 50 μM $HgCl_2$ in culture media for 10 min. After washing with PBS to remove the remaining mercury ions, the cells were incubated with 50 μM **1** in culture media for 20 min at 37 °C.

Alternatively, the neuron cells differentiated from PC12 cells were first incubated with 50 μM **1** in culture media for 20 min. After washing with PBS to remove the remaining sensor, the cells were then treated with 50 μM $HgCl_2$ for 10 min at 37 °C. The neuron cells incubated under both conditions were imaged using fluorescence microscopy.

Imaging of Human Muscle Precursor Cells Incubated with Mercury Ions and 1. The human muscle biopsy was generously provided by Dr. Hyun Woo Kim (Yonsei University College of Medicine). Human muscle fibers were isolated and cultured by the procedure developed by Rosenblatt et al.[20] Briefly, the muscle sample was digested in a Petri dish containing 0.2% collagenase (Sigma) in Ham's F10 media (Gibco) at 37 °C for 90 min and then washed with PBS several times. Most of the human muscle fibers were released from the tissue after enzymatic digestion. The single muscle fibers were isolated by repeatedly triturating the muscle fragments with a very wide-mouthed Pasteur pipet. Isolated muscle fibers were finally plated in 1 drop of medium Ham's F10 media into matrigel (Collaborative Biomedical Products, 1 mg/mL) coated 6-well plates (BioCoat) and allowed to attach for at least 4 h before adding plating media (10% FBS, 1% chick embryo extract (CEE, Sera Laboratories International) in Ham's-F10 media). After 4−5 days, the muscle precursor cells began to migrate from the fiber. The cells were counted by Trypsinisation and cultured in DMEM supplemented with 10% FBS, 1% CEE, 50 unit/mL of penicillin, and 50 μg/mL of streptomycin.

For mercury-ion uptake studies human muscle precursor cells were seeded in a 6-well plate at a density of 10^3 cells per well in culture media (DMEM supplemented with 10% FBS, 50 unit/mL of penicillin, and 50 μg/mL of streptomycin) and incubated with 50 μM $HgCl_2$ in culture media for 10 min. After washing with PBS to remove the remaining mercury ions, the cells were incubated with 50 μM **1** in culture media for 20 min at 37 °C.

Alternatively, human muscle precursor cells were incubated with 50 μM **1** in culture media for 20 min. After washing with PBS to remove the remaining sensor, the cells were treated with 50 μM $HgCl_2$ for 10 min at 37 °C. The cells were imaged using fluorescence microscopy.

Imaging of Zebrafish Incubated with Mercury Ions and 1. Zebrafish was kept at 28 °C and maintained at optimal breeding conditions. For mating, male and female zebrafish were maintained in one tank at 28 °C on a 12-h light/12-h dark cycle, and then the spawning of eggs was triggered by giving light stimulation in the morning. Almost all eggs were fertilized immediately. The 5-day-old zebrafish was maintained in E3 embryo media (15 mM NaCl, 0.5 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 0.15 mM KH_2PO_4, 0.05 mM Na_2HPO_4, 0.7 mM $NaHCO_3$, 10^{-5}% methylene blue; pH 7.5).[22] The 5-day-old zebrafish was incubated with 50 μM $HgCl_2$ in E3 media for 10 min at 28 °C. After washing with PBS to remove the remaining mercury ions, the zebrafish was further incubated with 50 μM **1** for 20 min at 28 °C.

Alternatively, the 5-day-old zebrafish was incubated with 50 μM **1** in E3 media for 20 min at 28 °C. After washing with PBS to remove the remaining sensor, the zebrafish was further incubated with 50 μM $HgCl_2$ for 10 min at 28 °C. The zebrafish was imaged by fluorescence microscopy and a dissecting microscope (Stemi 2000-C, ZAISS, Germany).

Real-Time Uptake of Mercury Ions by Mouse C2C12 Cells. C2C12 cells were seeded in a 96-well plate at a density of 10^3 cells per well in culture media. After 24 h, the culture media were replaced with fresh media and the cells were incubated with 200 μM **1** in culture media for 30 min. After washing with PBS to remove the remaining sensor, various concentrations (0−100 μM) of $HgCl_2$ were added to the cells in culture media, and fluorescence intensity was determined using a fluorescent microplate reader (SpectraMax GeminiEM, Molecular Devices).

Real-Time Uptake of Mercury Ions by 5-Day-Old Zebrafish. The 5-day-old zebrafish was incubated with 200 μM **1** in E3 media for 30 min at 28 °C. After washing with E3 media, various concentrations (0−100 μM) of $HgCl_2$ were added to the zebrafish in E3 media, and fluorescence intensity was determined by a fluorescent microplate reader.

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Supporting Information Available: Fluorescence response of **1** to Hg^{2+}, other metal ions, and CH_3HgCl, HPLC chromatograms, fluorescence intensity of cells treated with **1** and Hg^{2+}, and images of zebrafish organs treated with only **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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