Detection of mercury in fish organs with a two-photon fluorescent probe†

Chang Su Lim, Dong Won Kang, Yu Shun Tian, Ji Hee Han, Hye Lim Hwang and Bong Rae Cho*

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We report a two-photon fluorescent probe (AHg1) that can be excited by 780 nm femto-second pulses, shows high photostability and negligible toxicity, and can visualize the site of Hg^{2+} accumulation, but can also estimate trace amounts of mercury ions in fresh fish organs by two-photon microscopy.

Mercury is a notoriously toxic element that exists at 0.08 ppm in the Earth's crust.[1a] Every year, 2700–6000 tons of elemental mercury is released to the atmosphere through volcanic activity and 2000–3000 tons through coal combustion, gold production, and industrial waste.[1b] Once released, it can be converted to organic mercury by the bacteria in lakes and oceans and inorganic mercury in the liver. Three forms of mercury exist in the environment; elemental, inorganic, and organic, all of which are highly toxic. Mercury damages DNA, impairs mitosis, and disrupts the central nervous and endocrine systems.[1c]

The fish living in the polluted water can take Hg^{2+} through the mouth and skin, which can be transported to different organs. To prevent mercury-related problems, it is important to explore a new method of detecting trace amounts of Hg^{2+} in the fish organs in the early stage of contamination. For this purpose, a variety of water-soluble fluorescent probes have been developed.[2] Most of them employed fluorescein, rhodamine, and BODIPY as the fluorophore, and polyamide and thioether or a thioether-rich crown as the receptor. One of the most important achievements in this field is the development of one photon (OP) fluorescent probes that can distinguish safe and toxic levels of Hg^{2+} in edible fish samples.[2j,k,m,p] This method utilized the fluorescence analysis of the tissue samples digested under microwave in nitric acid. The efficiency and accuracy of such measurements could be significantly improved, if one could determine the Hg^{2+} concentration ($[Hg^{2+}]$) directly from the fish samples without further treatments. An ideal method for such a purpose is two-photon microscopy (TPM) in combination with a two photon (TP) probe for Hg^{2+}. TPM, which utilizes two photons of lower energy for the excitation, has the advantages of increased penetration depth ($\sim$500 μm), localized excitation, and prolonged observation time, thereby allowing intact tissue imaging.[3] However, TP probes for Hg^{2+} are rare.[4]

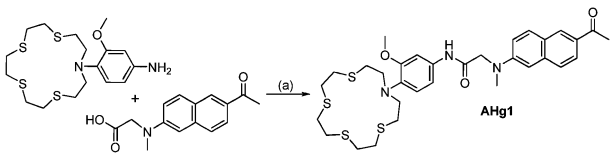
To design an efficient TP probe for Hg^{2+}, we considered the following requirements; (i) appreciable water solubility to stain the cells and tissues, (ii) significant TP cross section for bright TPM image, (iii) high selectivity for Hg^{2+}, (iv) a dissociation constant in the sub-ppm range to meet the US EPA standard for the upper level of Hg^{2+} in edible fish (0.55 ppm),[5] and (v) high photostability.

Herein, we report the synthesis and application of a TP probe for Hg^{2+} (AHg1) derived from a 6-dimethylamino-2-acetylnaphthalene (acedan) reporter and azathiocrown ether receptor that meets all of the requirements as outlined above. We chose acedan as the reporter because it has been successfully employed in various TP probes for metal ions, acidic vesicles, glucose, and membrane.[6] An azathiocrown ether receptor has been adopted from Chang's work for its high selectivity for Hg^{2+}.[2j,k] An *o*-methoxy group has been introduced to increase the binding ability of the receptor, as observed for the TP zinc ion probe (AZn2).[7]

Synthesis of AHg1 is shown in Scheme 1. *N*-(4-Amino-2-methoxyphenyl)-4,7,10,13-tetrathia-1-aza-15-crown-5 was prepared by the modified literature procedure.[2j] Coupling of the receptor moiety with 6-acyl-2-[*N*-methyl-*N*-(carboxymethyl)amino]naphthalene by *N*,*N*′-dicyclohexylcarbodiimide (DCC) produced AHg1 in 50% yield (see ESI† for details).

The water solubility of AHg1 was determined by plotting the fluorescence intensity *vs.* probe concentration as before.[6,7] AHg1 was soluble in water up to 4.0 μM, which is sufficient to stain the cells. The absorption and emission spectra of AHg1 showed gradual red shifts with the solvent polarity in the order, 1,4-dioxane < DMF < EtOH < H_2O. The large solvatochromic shifts with increasing solvent polarity indicate the utility of AHg1 as an environment-sensitive probe (Fig. S1 and Table S1, ESI†).

Spectroscopic measurements for AHg1 were performed under simulated physiological conditions (20 mM HEPES buffer, pH 7.0). AHg1 was weakly fluorescent (Φ = 0.025) and showed a 6-fold enhancement in the OP and TP fluorescence intensity [$FEF = (F - F_{min})/F_{min}$] upon addition of excess Hg^{2+} (Fig. 1a and Fig. S2a, ESI†), presumably because of the blocking of the photo induced electron transfer (PeT) by the complexation with the metal ion. The OP and TP



Scheme 1 Synthesis of AHg1. (a) DCC, HOBt, CH_2Cl_2.

Department of Chemistry, Korea University, 1-Anamdong, Seoul, Korea. E-mail: chobr@korea.ac.kr; Fax: (+82)2-3290-3544; Tel: (+82)2-3290-3129

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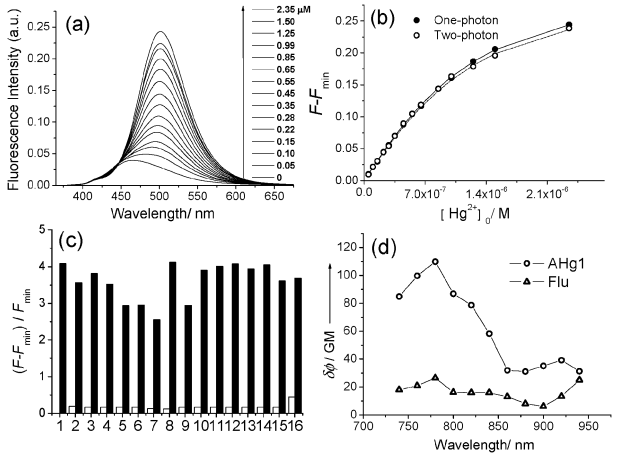

Fig. 1 (a) One-photon emission, and (b) one- (●) and two-photon (○) fluorescence titration curve for the complexation of AHg1 with Hg^{2+} (0–2.35 μM). (c) Relative fluorescence intensity of 1 μM AHg1 in the presence of 5 mM for Li^+, Na^+, K^+, Mg^{2+}, and Ca^{2+}, 300 μM for Fe^{2+}, Fe^{3+}, and Cu^+, and 500 μM for all other cations (empty bars) followed by the addition of 2.35 μM Hg^{2+} (filled bars). These data were obtained in 20 mM HEPES buffer (pH 7.0). (1) Hg^{2+}; (2) Li^+; (3) Na^+; (4) K^+; (5) Mg^{2+}; (6) Ca^{2+}; (7) Sr^{2+}; (8) Mn^{2+}; (9) Fe^{2+}; (10) Fe^{3+}; (11) Co^{2+}; (12) Ni^{2+}; (13) Cu^{2+}; (14) Zn^{2+}; (15) Cd^{2+}; (16) Pb^{2+}. (d) Two-photon action spectrum of AHg1 in the presence of Hg^{2+} (2.35 μM). The excitation wavelengths for one- and two-photon processes were 365 and 780 nm, respectively. The data for fluorescein was taken from ref. 6.

fluorescence titration curves for the complexation of AHg1 with Hg^{2+} showed linear increase up to 0.5 μM (0.1 ppm), indicating that AHg1 was suitable to detect Hg^{2+} in the sub-ppm range (Fig. 1b). The dissociation constants for AHg1 calculated from the fluorescence titration curves were $K_d^{OP} = 0.46 \pm 0.01$ μM and $K_d^{TP} = 0.45 \pm 0.01$ μM, for the OP and TP processes, respectively.[8] The values were smaller by 2-fold than that of Mercury Green 1 (MG1),[2k] indicating the enhanced binding ability that can be attributed to the electron-donating *o*-methoxy group.[7] Moreover, the linear Hill plot determined for Hg^{2+} binding with a slope of 1.0 indicated 1 : 1 complexation between AHg1 and Hg^{2+} (Fig. S2b in the ESI†).[9]

AHg1 showed excellent selectivity for Hg^{2+} over competing metal ions, as revealed by unperturbed fluorescence responses by millimolar concentrations of alkali and alkaline earth metal ions including Li^+, Na^+, K^+, Mg^{2+}, Ca^{2+}, Sr^{2+}, and 300–500 μM of first-row transition metal ions Mn^{2+}, Fe^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+}, group 12 ions Zn^{2+}, Cd^{2+}, and the common heavy-metal ion pollutant Pb^{2+}, as well as the dramatic increase in the fluorescence intensity upon addition of 1 equiv. (2.35 μM) of Hg^{2+} to the solutions containing the probe and competing ions (Fig. 1c).

The TP action spectrum of the AHg1–Hg^{2+} complex in the HEPES buffer indicated a TP action cross section (Φδ) value of 110 GM at 780 nm, a 3-fold larger than that of fluorescein (Φδ = 36 GM) (Fig. 1d).[7] This indicates that TPM images for samples stained with AHg1 would be brighter than those stained with fluorescein-based probes. Moreover, the spectral analysis of the emission spectra from the AHg1-labeled Henrietta Lacks (HeLa) cells indicated that the cytosolic

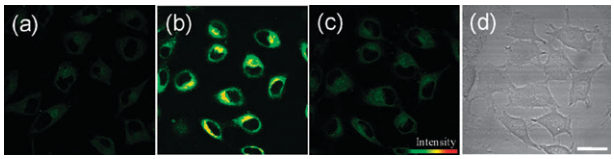

Fig. 2 (a–d) TPM image of HeLa cells labeled with AHg1 (2 μM) before (a) and after (b) addition of Hg^{2+} (20 μM). (c) TPM image of cells in panel (b) treated with the competing heavy-metal chelator TPEN (1 mM) for an additional 5 min at 25 °C. (d) Bright-field image of cells in panel (c). The TPEF was collected at 500–620 nm upon excitation at 780 nm with fs pulse. Scale bars, 30 μm. Cells shown are representative images from replicate experiments ($n = 5$).

Hg^{2+} can be selectively detected by using the detection window of 500–620 nm, with minimum interference from the membrane-bound probes (Fig. S3 in the ESI†). Further, the TP excited fluorescence (TPEF) intensity at a given spot on the AHg1-labeled HeLa cells remained nearly the same after continuous irradiation of the fs-pulses for 60 min, indicating its high photostability (Fig. S4, ESI†).

To explore the utility of this probe, we have tested the ability of AHg1 to detect the Hg^{2+} in live cells (Fig. 2). The TPM image of the HeLa cells labeled with 2 μM of AHg1 for 20 min at 37 °C shows weak background emission (Fig. 2a), consistent with the efficient fluorescence quenching by PeT (*vide supra*). The TPEF increased significantly when the cells were exposed to 20 μM of Hg^{2+} for 20 min (Fig. 2b), and decreased to the base level upon treatment with 1 mM *N*,*N*,*N*′,*N*′-tetrakis(2-pyridyl)ethylenediamine (TPEN), a membrane-permeable heavy metal ion chelator that can effectively remove Hg^{2+} (Fig. 2c). The bright field image shown in Fig. 2d and the image of HeLa cells stained with Hoechst 33·342 confirm the cell viability (Fig. S5 in the ESI†). Hence, AHg1 is clearly capable of detecting Hg^{2+} in live cells.

We also investigated AHg1 to trace where Hg^{2+} is accumulated in a fish. For this experiment, 100 *Oryzias latipes*, approximately 5 month post-hatched and fully mature with an average body weight of 0.28 g and 3.0 cm long (see ESI†), were acclimated in water tanks for one week. The fish were divided into two groups, half of them in aquaria containing 2 ppb of $HgCl_2$ and the other half in aquaria without $HgCl_2$, and reared for 1 and 3 days, according to the OECD guideline for testing of chemicals in a fish.[10] All fish survived because the Hg^{2+} concentration was much lower than the LC_{50} value of 700 ppb.[11] The fish were euthanized, and kidney, heart, gill, and liver, where Hg^{2+} is known to be accumulated,[12] were dissected. The TPM images of the AHg1-labeled organs obtained at different depths show that the Hg^{2+} is almost evenly distributed along the *z* direction (Fig. S6 in the ESI†). Moreover, the images obtained at a higher magnification clearly revealed the site of Hg^{2+} accumulation, that is, glomerulus of the kidney, blood vessels in the heart, hepatocyte in the liver, and gill filaments of the gill (Fig. 3). These results demonstrate that AHg1 is capable of tracing the sites of Hg^{2+} accumulation in the fresh fish.

To further demonstrate the utility of this probe, we have determined the Hg^{2+} concentration in each organ by TPM. For each organ, the average TPEF intensity from 70 TPM images, 7 TPM images in the *xy* plane along the *z*-direction of

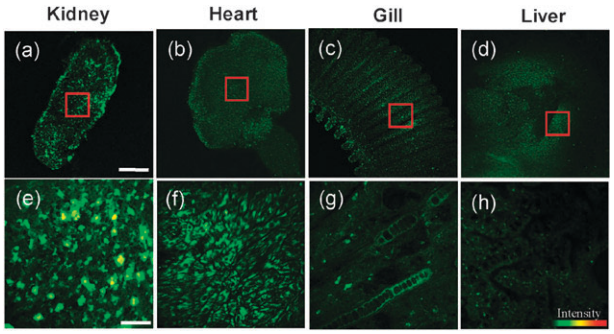

Fig. 3 (a–d) TPM images of kidney, heart, gill, and liver of *Oryzias latipes* obtained at 100 μm depth by magnification at 10×. (e–h) The regions indicated by the red boxes in a–d are magnified at 100×. All organs were stained with 10 μM AHg1 and the TPM images were obtained by collecting the TPEF at 500–620 nm upon excitation at 780 nm with fs pulses. Scale bar, 300 (a–d) and 30 μm (e–h).

10 organ samples, was determined. The TPEF intensity increased with the exposure time, and the highest intensity was observed in the kidney, followed by heart, gill, and liver (Fig. 4). This indicates that Hg^{2+} is accumulated in the order, kidney > heart > gill ≥ liver. The Hg^{2+} concentration in the kidney and heart after 1 and 3 days exposure, estimated from the linear region in Fig. 1b and the TPEF intensity, were 32 and 40 ppb and 15 and 25 ppb, respectively. For comparison, the Hg^{2+} concentration in the kidney after 3 days of exposure determined by inductively coupled plasma mass spectrometry (ICP MS) was 42 ppb, which was in excellent agreement with the measured value by TPM. These results indicate that AHg1 is capable of estimating the $[Hg^{2+}]$ in fresh fish organs by TPM at much lower concentrations than 0.55 ppm, the upper level of mercury in edible fish according to US EPA standards.[5] Therefore, AHg1 should find utility in detecting trace amounts of Hg^{2+} in fish organs in the early stage of contamination.

To conclude, we have developed a TP probe (AHg1) that shows 6-fold TPEF enhancement in response to Hg^{2+}, a

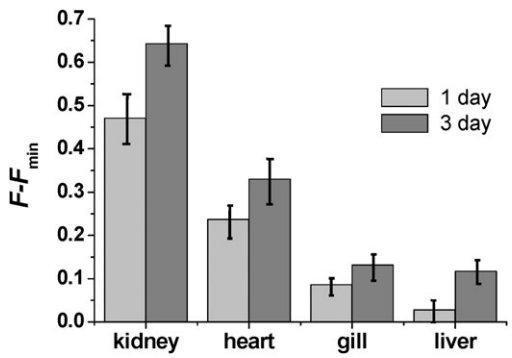

Fig. 4 Relative TPEF intensity in kidney, heart, liver and gill of *Oryzias latipes* after exposure to 2 ppb Hg^{2+} for 1 and 3 days. The organs were incubated with AHg1 (10 μM) for 30 min and the TPEF was collected at 500–620 nm upon excitation with fs pulses at 780 nm. The columns and error bars represent the average and standard deviation of the TPEF intensities from 70 TPM images.

dissociation constant (K_d^{TP}) of 0.45 ± 0.01 μM, and can selectively detect Hg^{2+} in live cells and fish organs at 80–150 μm depth by TPM without interference from other metal ions or membrane bound probes. Better than the currently available probes, this novel probe can not only visualize the site of Hg^{2+} accumulation, but also estimate trace amounts of $[Hg^{2+}]$ in fresh fish organs by TPM.

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