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A Two-Photon Probe for Hg(II) in Fish Organs†

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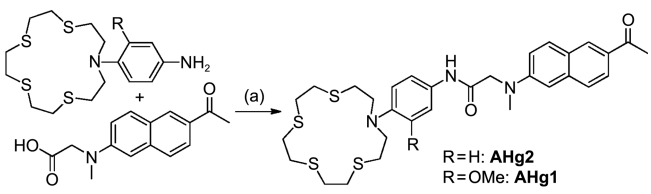
Mercury is a toxic and hazardous metal that damages DNA, impairs mitosis, and disrupts the central nervous and endocrine systems.[1] Recently, we reported a two photon (TP) probe for Hg^{2+} (**AHg1**) that can detect Hg^{2+} in the fish organs by two-photon microscopy (TPM). However, **AHg1** showed modest (6-fold) enhancement in the TP fluorescence intensity upon binding with Hg^{2+} and dissociation constant too small to detect toxic levels of Hg^{2+} in a fish.[2] To distinguish safe and toxic levels of Hg^{2+} in edible fish by TPM, we have now developed a new TP probe (**AHg2**) in which the *o*-OMe in **AHg1** is replaced by H, with the expectation that the nitrogen atom in the receptor moiety would become less basic due to the efficient resonance interactions with ppp-orbitals, thereby decreasing the Hg^{2+} affinity. **AHg2** was prepared as shown in Scheme 1 (See Supporting Information (SI) for details).

The water solubility of **AHg2** is 4.5 μM in HEPES buffer ([HEPES] = 20 mM, pH 7.0), which is sufficient for cellular staining (Figure S2).[3] The absorption and emission spectra of **AHg2** 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 **AHg2** as the environment-sensitive probe (Figure S1 and Table S1, SI).

When small increments of Hg^{2+} were added to **AHg2** in HEPES buffer, one- and two-photon excited fluorescence intensity increased gradually without affecting the absorption spectra, presumably because of the blocking of the photo induced electron transfer (PeT) by the complexation with the metal ion. The fluorescence enahncement factors [$FEF = (F - F_{min})/F_{min}$] measured for one- and two-photon processes were 60 in the presence of excess Hg^{2+} (Figure 1a). The one-photon (OP) and TP fluorescence titration curves for the complexation of **AHg2** with Hg^{2+} showed linear increase up to 10 μM (2.0 ppm), indicating that **AHg2** is suitable to



Scheme 1. Synthesis of **AHg2**. (a) DCC, HOBt, CH_2Cl_2.

†This paper is dedicated to Professor Eun Lee on the occasion of his honourable retirement.

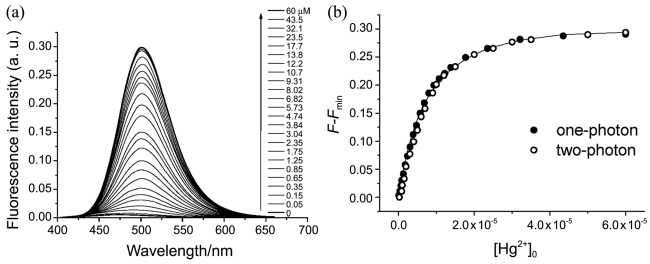


Figure 1. (a) One-photon emission, and (b) one- (●) and two-photon (○) fluorescence titration curve for the complexation of **AHg2** (1.0 μM) with Hg^{2+} (0-60 μM).

detect Hg^{2+} in the ppm range (Figure 1b). The dissociation constants for **AHg2** calculated from the fluorescence titration curves are $K_d^{OP} = 5.0 \pm 0.1$ μM and $K_d^{TP} = 5.1 \pm 0.1$ μM, for the OP and TP processes, respectively.[4] The values are smaller by 10-fold than that of **AHg1**,[2] indicating its ability to detect higher concentration of Hg^{2+}. Moreover, the titration curve fitted well with 1:1 binding model (Figure 1b) and the Hill plots were linear with a slope of 1.0 (Figure S2b), indicating 1:1 complexation between the probe and Hg^{2+}.[5]

AHg2 exhibited high 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+}, and Ca^{2+}, 300-500 μM of first-row transition metal ions Sr^{2+}, 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 (60 μM) of Hg^{2+} to the solutions containing the probe and competing ions (Figure S2c).

The TP action spectrum of the **AHg2**-Hg^{2+} complex in the HEPES buffer indicated a Fd value of 78 GM at 750 nm, a value comparable to that of **AHg1**-Hg^{2+} complex (Figure S2d).[2] This predicts a bright TPM image of the samples stained with **AHg2**. Moreover, the two-photon excitation fluorescence (TPEF) intensity at a given spot on the **AHg2**-labeled HeLa cells remained nearly the same after continuous irradiation of the fs-pulses for 60 min (Figure S3). These results establish the capability of **AHg2** for detecting Hg^{2+} by TPM for a long period of time with minimum interference from other competing metal ions. Further, the TPEF spectra of the HeLa cells labeled with 2 μM **AHg2** were nearly the same before and after treatment with Hg^{2+} and *N,N,N',N'*-tetrakis (2-pyridyl)ethylenediamine (TPEN), a membrane-permeable

heavy metal ion chelator that can effectively remove Hg^{2+}, with emission maxima at 480 nm (Figure S4). Therefore, we have collected TPEF in the 400-620 nm range.

We next sought to apply **AHg2** as a TP probe for Hg^{2+} in live cells (Figure 2). The TPM image of the HeLa cells labeled with 2 μM of **AHg2** for 20 min at 37 °C shows little background emission (Figure 2a), consistent with the efficient fluorescence quenching by PeT (*vide supra*). The TPEF increased significantly when the cells were exposed to 50 μM of Hg^{2+} for 20 min (Figure 2b), and decreased to the base level upon treatment with 1 mM TPEN (Figure 2c). The bright field image shown in Figure 2d and the image of HeLa cells stained with Hoechst 33342 confirm the cell viability (Figure S5). Hence, **AHg2** is clearly capable of detecting Hg^{2+} in the live cells.

We then investigated **AHg2** to trace where Hg^{2+} is accumulated in a fish. For this experiment, 100 *Oryzias latipes* were devided into two groups, half of them in aquaria containing 20 ppb of $HgCl_2$ and the other half in aquaria without $HgCl_2$, and reared for 3 days, according to the OECD guideline for testing of chemicals in a fish.[6] The fishes were euthanized, and kidney, heart, gill, and liver, where Hg^{2+} is known to be accumulated,[7] were dissected. The TPM images of the **AHg2**-labeled organs 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 (Figure 3). Moreover, the TPM images at the depths 80, 110, 140, and 170 μm show the Hg^{2+} distribution in each *xy* plane along the *z* direction (Figure S6). These results establish that **AHg2** is capable of tracing the sites of Hg^{2+} accumulation in the fresh fish.

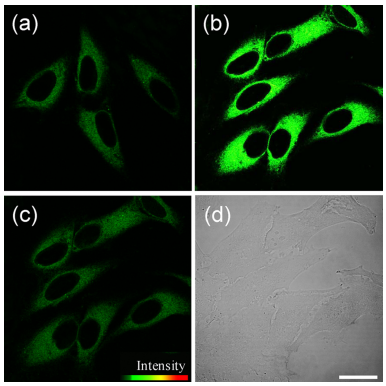


Figure 2. (a-d) TPM image of HeLa cells labeled with **AHg2** (2 μM) before (a) and after (b) addition of Hg^{2+} (50 μM). (c) TPM image of cells in panel (b) treated with the competing heavy-metal chelator TPEN (1 mM) for an additional 10 min at 25°C. (d) Bright-field image of cells in panel (c). The TPEF was collected at 400-620 nm upon excitation at 750 nm with fs pulse. Scale bars, 30 μm. Cells shown are representative images from replicate experiments (n = 5).

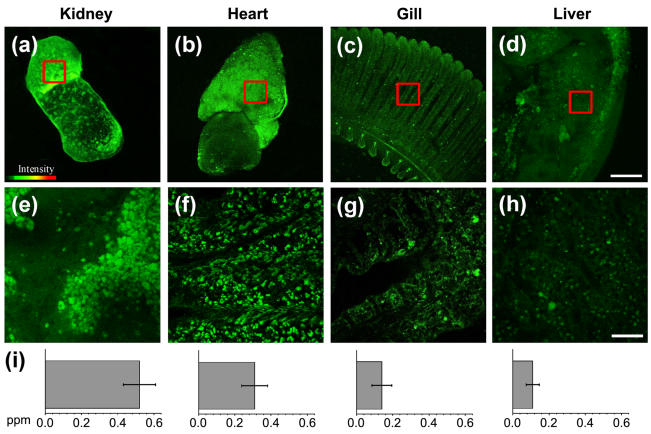


Figure 3. TPM images of kidney, heart, gill, and liver of *Oryzias latipes* obtained at 100 μm depth by magnification at 10×. (e-h) The regions indicated by the red boxes in a-d are magnified at 100×. (i) The Hg^{2+} contents in the red boxes in a-d. All organs were stained with 10 μM **AHg2** and the TPM images were obtained by collecting the TPEF at 400-620 nm upon excitation at 750 nm with fs pulses. Scale bar, 300 (a-d) and 30 μm (e-h).

To further establish the utility of **AHg2**, we have estimated the Hg^{2+} contents in each organ by TPM. TPM image was brightest in the kidney, followed by heart, gill, and liver, that is, Hg^{2+} is accumulated in the order, kidney > heart > gill ≥ liver (Figure 3). The image obtained at a higher magnification clearly reveals the Hg^{2+} distribution at 100 μm depth. Moreover, the Hg^{2+} contents in each organ, estimated from the calibration curve obtained by plotting the TPEF intensity of **AHg2** (10 μM)-labeled cells vs [Hg^{2+}] (Figure S7) and F-F_{min} values, are 0.52, 0.31, 0.14, and 0.11 ppm, respectively (Figure 3i). This result indicates that **AHg2** can detect [Hg^{2+}] in fresh fish organs by TPM at 0.1-0.5 ppm level, less than the upper level of Hg^{2+} (0.55 ppm) in edible fish according to US EPA standard.[8] Therefore, **AHg2** should find utility in distinguishing the safe and toxic level of Hg^{2+} in the fish organs by TPM.

To conclude, we have developed a TP probe (**AHg2**) that shows 60-fold TPEF enhancement in response to Hg^{2+}, a dissociation constant (K_d^{TP}) of (5.1 ± 0.01) μM, and can selectively detect Hg^{2+} in live cells and fish organs at 80-170 μm depth by TPM without interference from other metal ions. This novel probe can not only visualize the site of Hg^{2+} accumulation, but also distinguish safe and toxic levels of Hg^{2+} in fresh fish organs by TPM.

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