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Reaction-Based Two-Photon Probes for Mercury Ions: Fluorescence Imaging with Dual Optical Windows

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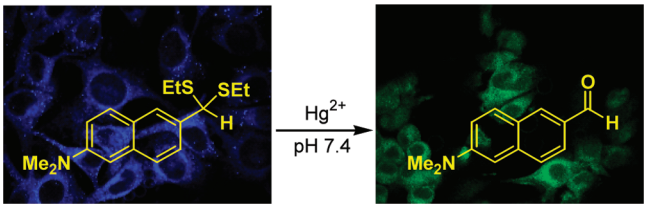
ABSTRACT



For fluorescent imaging of mercury ions in living species, two-photon probes with dual optical windows are in high demand but remain unexplored. Several dithioacetals were evaluated, and a probe was found, which, upon reaction with mercury species, yielded a two-photon dye; this conversion accompanies ratiometric emission changes with a 97-nm shift, enabling fluorescent imaging of both the probe and mercury ions in cells by one- and two-photon microscopy for the first time.

Mercury is an indispensable element in the current chemical industry, yet the highly poisonous nature of mercury and its oxidized species have drawn our keen concern regarding their environmental contamination.[1] Efficient detection and monitoring tools for mercury species are thus essential for assessing their contamination of the environment and living species. Among various detection tools for mercury species, fluorescent probes have gained significant attention owing to their sensitive and easy-to-use features, in addition to their usefulness in bioimaging.

Efforts to develop efficient fluorescent probes for mercury species resulted in various types of probes.[2] Along with the supramolecular approach, the reaction-based approach, which utilizes a mercury-specific chemical conversion, has received much attention recently for the realization of high analyte selectivity and turn-on type fluorescence change.[3] In particular, sulfur-to-oxygen exchange reactions have received widespread attention. Recently, by choosing a new class of mercury-promoted reactions, that is, the hydrolysis of aryl vinyl ethers, we were able to fluorescently image methylmercury in living species for the first time.[4]

For the application of fluorescent probes in the imaging of mercury species in cells and organs, first we need to know which part of a living species the probe distributes to, before sensing the presence of mercury ions in that part. In this context, a desirable probe is the one that enables us to monitor the probe itself and its interaction outcome with mercury ions through separate optical windows (probe−analyte dual probing). The fluorescence resonance

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(1) (a) ATSDR 1999. Toxicological Profile for Mercury: Atlanta, GA, U.S. Department of Health and Human Services. (b) ATSDR 2005. ToxProfiles: Mercury: Atlanta, GA, U.S. Department of Health and Human Services.

(2) For selected reviews, see: (a) Nolan, E. M.; Lippard, S. J. *Chem. Rev.* **2008**, *108*, 3443. (b) Kim, H. N.; Ren, W. X.; Kim, J. S.; Yoon, J. *Chem. Soc. Rev.* **2012**, *41*, 3210.

(3) For a recent review on reactive probes, see: Jun, M. E.; Roy, B.; Ahn, K. H. *Chem. Commun.* **2011**, *47*, 7583.

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energy transfer systems satisfy this requirement, but they require multistep synthesis in general. In contrast, most of the ratiometric systems based on the internal charge transfer phenomenon lack such ability owing to severe spectral overlap.

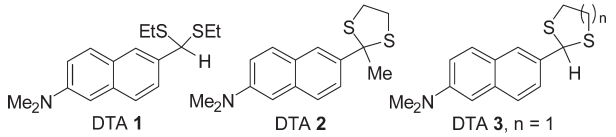
In addition to the dual probing property, how to secure deep-tissue imaging is also an important concern when we apply a fluorescent probe to the imaging of mercury ions in living species. A two-photon (TP) probe is promising for this purpose, as with it we can look into deeper tissue compared with the conventional one-photon-excitation probes.[5] Furthermore, two-photon probes alleviate other unfavorable features one-photon probes may cause in bioimaging such as photobleaching, photodamage, and cellular autofluorescence.

So far, only a few TP probes for mercury ions are known, but most of them show limitations such as fluorescence quenching toward mercury ions,[6a] poor water solubility,[6b] and low fluorescence enhancement.[6c] Furthermore, dual probing behavior has not been demonstrated by any of these sensing systems.

Herein, we report a novel sensing system for mercury ions that satisfies the desirable properties discussed above. The probe thus enabled us to separately image the probe alone and the presence of mercury species in cells by one-photon microscopy (OPM) as well as two-photon microscopy (TPM) for the first time.

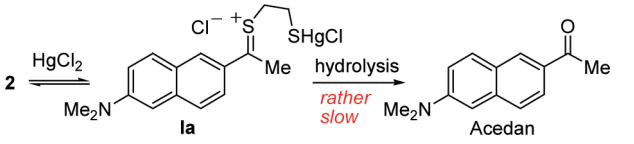
The two-photon probe we developed throughout this study is DTA **1** (Scheme 1). It is structurally simple yet shows the desirable two-photon property as well as the dual probing ability. Initially we examined DTA **2**, a dithioacetal of 1-(6-dimethylamino-naphthalen-2-yl)ethanone (acedan), as a potential TP probe for mercury ions because acedan is a well-established two-photon fluorophore (Scheme 2).[7] Ketone [1,3]dithiolanes such as DTA **2** are known to be stable under basic, neutral, and mildly acidic conditions but readily undergo mercury ion-promoted hydrolysis, a well-established deprotection process.

Scheme 1. Dithioacetals **1**–**4** Evaluated As Two-Photon Probes for Mercury Ions



Because the mercury ion-promoted hydrolysis of DTA **2** should regenerate acedan, it would allow us to detect and image mercury ions by OPM as well as TPM. Our keen concern with this sensing strategy was whether the deprotection process would accompany the desirable emission changes within a reasonable reaction time.

Scheme 2. Mercury Ion-Promoted Hydrolysis of Dithioacetal **2** via a Sulfonium Intermediate



A slight change in the UV–vis absorption spectra was observed after a few minutes upon mixing DTA **2** (10 μM) with 1 equiv of Hg^{2+} (as chloride salt) in pH 7.4 buffer (10 mM HEPES containing 1% CH_3CN) at room temperature (Figure S1); this result suggests that the Hg^{2+}-promoted hydrolysis is not fast in the case of DTA **2**. When the solution was incubated for 1 h, the absorption band at 300 nm decreased and the broad band at 370 nm increased owing to the hydrolysis process. DTA **2** exhibited weak fluorescence at 430 nm when excited at 330 nm; however, upon addition of Hg^{2+}, a new emission band at 516 nm increased gradually and saturated after about 30 min. The fluorescence increase, however, involved abrupt changes in the initial stage (Figures S2 and S3), which may be explained by a slow hydrolysis of the initial reaction intermediate **Ia**, as the tethered thiolate group can readily reattack the sulfonium carbonyl and return to DTA **2** (Scheme 2). The intermediate **Ia** may also give fluorescence owing to the internal charge transfer (ICT), together with its hydrolysis product, acedan. Coexistence of these two fluorescent species thus seems to be responsible for the abrupt changes in the initial titration stage.

To improve the low reactivity of cyclic dithioacetal **2**, we turned our attention to aldehyde dithioacetal **1** based on the fact that aldehyde acetals, in general, undergo the Lewis acid promoted hydrolysis reaction faster than the corresponding ketone acetals. The hydrolysis product (aldehyde **5**),[8] which is structurally similar to acedan, is also expected to show two-photon absorbing behavior. Furthermore, aldehyde dithioacetal **1** shows sufficient stability for our sensing purpose (Figure S4). Related cyclic dithioacetals **3** and **4** were also evaluated in comparison with **1**, with respect to the reactivity and emission behavior. It should be noted that dithiane compounds were developed as colorimetric sensing system for mercury ions.[9]

An efficient synthetic route to DTAs **1**–**4**, starting from 6-bromo-naphth-2-ol, has been established. Using a

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Bucherer reaction,[10] the hydroxyl group of the naphthol was substituted with a dimethylamino group in the presence of the 6-bromo substituent in good yield (Schemes S1 and S2).

As expected, DTA **1** underwent a fast mercury-promoted hydrolysis, giving the product **5** within a few minutes in pH 7.4 buffer at room temperature (Figures S1 and S2). Monitoring the hydrolysis reaction by ^{1}H NMR showed the immediate appearance of the aldehyde proton (δ 10 ppm) of **5** with the concomitant disappearance of the methine proton (δ 5.2 ppm) of DTA **1**, together with other protons shifted downfield (Figure S5).

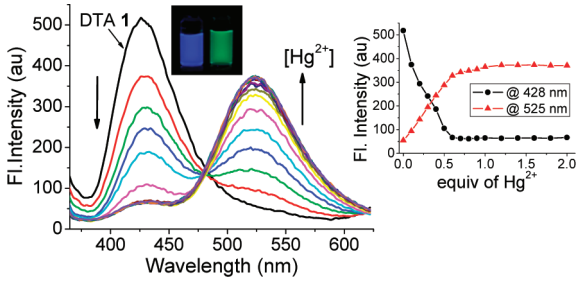
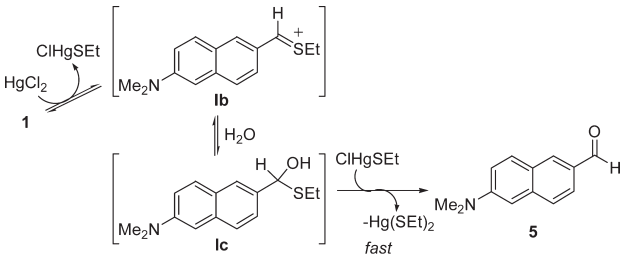


Figure 1. Fluorescence changes of DTA **1** (10 μM) upon addition of Hg^{2+} (0–2 equiv) in pH 7.4 buffer (10 mM HEPES containing 1% CH_3CN), measured after 3 min (λ_{ex} = 330 nm). Photograph of DTA **1** (10 μM) in the absence (left) and presence (right) of Hg^{2+} (1 equiv) in the buffer, taken under excitation at 356 nm. Inset: plot of the intensity maximum values at 428 and 525 nm, respectively, depending on the molar equiv of Hg^{2+} with respect to DTA **1**.

Under lower concentration conditions used for UV–vis spectroscopy, a fast hydrolysis was still indicated: An equimolar solution of DTA **1** (10 μM) and Hg^{2+} in pH 7.4 buffer showed a new absorption band at 369 nm owing to **5** generated through the hydrolysis, with simultaneous disappearance of the absorption band of DTA **1** at 311 nm, within a few minutes at room temperature (Figure S6). Interestingly, DTA **1** itself showed strong fluorescence with λ_{em} at 428 nm, in contrast to DTA **2**. Upon addition of 1 equiv of Hg^{2+} to DTA **1** in pH 7.4 buffer, a new emission band at 525 nm appeared while the probe's band disappeared within a few minutes (λ_{ex} = 330 nm) (Figure S6). Fluorescence titration of DTA **1** (10 μM) with varying amounts of Hg^{2+} (0–2 equiv) in pH 7.4 buffer thus shows ratiometric behavior (Figure 1). Of particular note is that the two emission bands show little spectral overlap with a significant wavelength shift ($\Delta\lambda_{max}$ = 97 nm), from which we expect that DTA **1** can be used for bioimaging through dual optical windows—one for the probe and the other for the product **5**.

Scheme 3. Mechanism of Hg^{2+}-Promoted Conversion of DTA **1** to Aldehyde **5** with 1 equiv of $HgCl_2$



It should be noted that only DTA **1** shows such good ratiometric behavior, and other cyclic dithioacetal analogues, **3** and **4**, do not (Figure S2 and S7). As noted, DTA **2** shows a turn-on type response toward Hg^{2+} albeit it is rather slow.[11] Dithioacetals **3** and **4** seem to undergo the hydrolysis very slowly under the sensing conditions, as inferred from very weak fluorescence observed even after 1 h; at this point, the absorption spectra corresponding to the dithioacetals significantly decreased. The absorption and emission spectral changes suggest that, for such cyclic acetals, the sulfonium intermediates corresponding to the intermediate **Ia** form within 1 h but persist and do not produce the product **5** readily.

According to the hydrolysis mechanism in the case of DTA **1** (Scheme 3), an organomercury species, ClHgSEt, also seems to promote the hydrolysis reaction. Indeed, separate titration data (Figure S8) clearly showed that 0.5 equiv of Hg^{2+} could induce complete hydrolysis. Although we have not examined methylmercury because of its notoriously toxic nature, the sensing ability of DTA **1** toward the alkylthiomercury indicates that the probe can also be used for the detection of methylmercury.[12] This reactivity seems to be another advantageous feature of such dithioacetal-based probes.[11]

Next we have evaluated the probe's selectivity toward various metal salts. DTA **1** showed distinct ratiometric behavior in the absorbance and emission spectra toward Hg^{2+} over the competing metal ions (Li^+, Na^+, K^+, Ba^{2+}, Ca^{2+}, Mg^{2+}, Zn^{2+}, Ni^{2+}, Pt^{2+}, Sn^{2+}, Co^{2+}, Mn^{2+}, Cd^{2+}, Cr^{2+}, Cu^{2+}, Pb^{2+}, Al^{3+}, and Fe^{3+}) except Ag^+ (Figure S9). Competition with Ag^+ to sulfur-based Hg^{2+} probes is not unexpected.[6b,13] DTA **1** may be used as TP probe for Ag^+, even though it is less reactive than the case of Hg^{2+} (Figure S10). A competitive metal screening also confirmed the selective response of DTA **1** toward the competing ions (Figure 2).

The detection limit of DTA **1** was estimated to be 3 ppb based on the linear relationship between the mercury concentration (1–100 ppb) and the corresponding fluorescence enhancement data (Figure S11).

(11) During preparation of this manuscript, a 1,3-dithiane analogue of **2** appeared as a turn-on type fluorescent probe for mercury ions with TP imaging data but with a very long incubation time: Huang, R.; Zheng, X.; Wang, C.; Wu, R.; Yan, S.; Yuan, J.; Weng, X.; Zhou, X. *Chem.—Asian J.* **2012**, *7*, 915.

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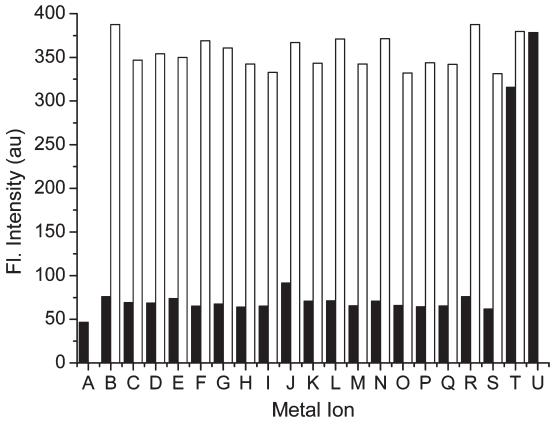

Figure 2. Fluorescence changes (peak height at 525 nm) of DTA **1** (10 μM) upon addition of each metal ion (50 μM, filled bars) followed by Hg^{2+} (50 μM, empty bars) in pH 7.4 buffer (10 mM HEPES containing 1% CH_3CN), measured after 3 min (λ_{ex} = 330 nm): (A) DTA **1**, (B) Li^+, (C) Na^+, (D) K^+, (E) Ba^{2+}, (F) Ca^{2+}, (G) Mg^{2+}, (H) Zn^{2+}, (I) Ni^{2+}, (J) Pt^{2+}, (K) Sn^{2+}, (L) Co^{2+}, (M) Mn^{2+}, (N) Cd^{2+}, (O) Cr^{2+}, (P) Cu^{2+}, (Q) Pb^{2+}, (R) Al^{3+}, (S) Fe^{3+}, (T) Ag^+, and (U) Hg^{2+}.

To demonstrate the usefulness of the probe, we have applied it to the fluorescent imaging of Hg^{2+} present in live cells. The probe showed little cytotoxicity to B16F10 mouse skin cancer cells up to a level of 100 μM for 1 h from a cell counting kit (CCK-8) assay (Figure S12). Fluorescence imaging of Hg^{2+} in B16F10 mouse skin cancer cells was carried out by bright-field and OPM (Figure S13) as well as by TPM (Figure 3). Two groups of cells were exposed to DTA **1** (50 μM) for 30 min and then washed three times with PBS buffer to remove excess probe, and one group of cells were incubated with Hg^{2+} (100 μM) for 30 min and then washed with PBS buffer to remove unreacted Hg^{2+}. OPM and TPM images were recorded for these cell groups using appropriate filters (OPM: for DTA **1**, 350−390 nm for excitation and 450−470 nm for emission; aldehyde **5**, 460−500 nm for excitation and 500−550 nm for emission. TPM: for both DTA **1** and aldehyde **5**, excitation at 730 nm; for emission of DTA **1**, 430−480 nm; for emission of aldehyde **5**, 500−550 nm). The blue OPM image taken for the cells

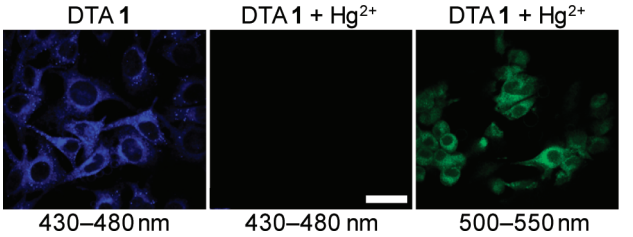

Figure 3. TPM images of B16F10 mouse skin cancer cells labeled with DTA **1** (50 μM), before and after addition of Hg^{2+} (100 μM). Excitation at 730 nm; emission filters used are shown below the images. Scale bar: 30 μm.

incubated with DTA **1** indicates that the probe readily penetrates the cancer cells (Figure S13). The cells treated with Hg^{2+} show green fluorescence owing to the hydrolysis product **5**. The cells treated with the probe alone also show bright blue fluorescence by TPM, which, upon treatment with Hg^{2+}, gives bright green fluorescence owing to **5** (Figure 3). When the optical filters were used for the probe, no blue fluorescence image was observed for the cells treated with both the probe and Hg^{2+}; this result indicates that the sensing process in the cells is complete. The results demonstrate that DTA **1** enables fluorescent imaging of both the probe and Hg^{2+} in cells for the first time.

In conclusion, we have developed a reaction-based two-photon probe for mercury species. The probe, a dithioacetal of a two-photon absorbing aldehyde, undergoes a fast mercury ion-promoted deprotection to yield the aldehyde. The conversion accompanies a ratiometric emission change with a significant wavelength shift, enabling fluorescent imaging of both the probe and mercury ions in cells by one- and two-photon microscopy for the first time.

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Supporting Information Available. Synthesis of new compounds, optical spectra of DTA **1**−**4** and experimental for one- and two-photon microscopy. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.