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Highly Selective Cysteine Detection and Bioimaging in Zebrafish through Emission Color Change of Water-Soluble Conjugated Polymer-Based Assay Complex

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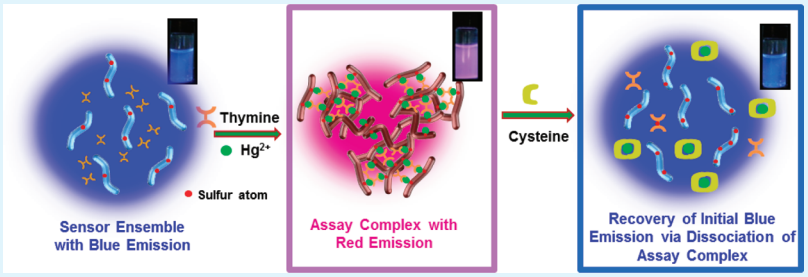
Supporting Information



ABSTRACT: A new concept for rapid, label-free cysteine sensing method is proposed via possible naked eye-detection of red-to-blue emission color change. Intermolecular exciton migration in conjugated polyelectrolyte-based assay complex is adopted to enhance selectivity and sensitivity for cysteine sensing by formation and dissociation of polymer−Hg^{2+}−thymine assay complex in the absence and presence of cysteine, respectively. The assay complex shows red emission due to cooperative aggregation of conjugated polyelectrolyte, thymine, and Hg^{2+}. Upon exposure to cysteine, the assay complex dissociates into individual molecules showing transparent, blue-emitting solution, because cysteine extracts Hg^{2+} from the assay complex via more favorable binding between cysteine and Hg^{2+}.

KEYWORDS: *conjugated polymers, sensors, fluorescence, cysteine, emission color change, polyelectrolyte, thymine, bioimaging*

1. INTRODUCTION

Cysteine (Cys) is a nonessential, sulfur-containing amino acid that can be found in many proteins throughout the human body.[1] Because it is one of the few amino acids that contain sulfur, Cys serves to bond in a special way through disulfide bonds and support the secondary structure of proteins in the human body.[2] Therefore, Cys deficiency is related to many syndromes such as the slow growth of cells, edema, liver damage, hair depigmentation, skin lesions, and weakness and has a bad influence on health, leading to problems such as metabolic disease or malabsorption syndromes.[3] Thus, the quantitative detection of Cys is extremely important for human healthcare.

A wide variety of detection methods for Cys using various materials and mechanisms have been developed such as fluorescence-coupled HPLC,[4a,b] electrochemical methods,[4c−e] and fluorometric analysis based on fluorescent sensors.[5] Among the various reported methods for the determination of Cys, fluorescence sensing methods are known to be convenient and simple to operate and, thus, can be used for intercellular bioimaging analysis.[6]

Despite the development of many fluorescent sensors for Cys, the detection of biological Cys in aqueous media is still challenging.[7] Furthermore, most of the recently reported fluorescent sensors exhibit only changes in the fluorescence intensity, which might be misleading in quantitative detection due to many environmental factors.[8] In contrast, fluorescent sensors that exhibit fluorescence color changes upon binding to an analyte are attractive, because naked-eye detection is possible resulting from their emission color change.

Fluorescent conjugated polymers have been widely used as sensitive chemosensors for detecting a variety of analytes due to

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the signal amplification effect via molecular wire effect of the conjugated polymer backbone.[9] Among these polymers, water-soluble conjugated polymers with ionic charges, namely, conjugated polyelectrolytes, can be used to amplify fluorescence signals in aqueous solution and have drawn a great deal of attention as fluorescent biomolecular sensory materials.[10]

Fluorescent conjugated polyelectrolytes, which exhibit different fluorescence colors in aqueous solution (short wavelength emission) and in the solid state (long wavelength emission) have been reported.[11] This phenomenon results from the unique aggregation-induced energy transfer or aggregation-induced intermolecular exciton migration and has been used for protein sensing with a noticeable blue-to-green or blue-to-red emission color change. The sensing mechanism was also adopted to design a new fluorescent sensor ensemble for the simultaneous sensing and removal of the Hg^{2+}. With the above-mentioned conjugated polyelectrolyte in the presence of thymine (T), Hg^{2+} can be detected with a blue-to-red emission color change and effectively removed via aggregation of the sensor ensemble.[12a]

Herein, a new concept for a label-free fluorescent Cys-detecting system based on aggregation-induced fluorescence color changes is described, in which an assay complex, composed of conjugated polyelectrolyte **1**, Hg^{2+}, and T, was employed to enhance the sensitivity and selectivity. It was previously reported that Cys can bind Hg^{2+} strongly to form a complex of Hg^{2+} and sulfur atom with ratio of 1:2.[13] We conjectured that the assay complex of the Hg^{2+}-containing sensor system (**1**-Hg^{2+}-T) could be easily dissociated by the addition of Cys (**1**,T, and Hg^{2+}-Cys), which should show a phase change of aggregates-to-solution leading to a red-to-blue emission color change. The assay of the **1**-Hg^{2+}-T complex has the advantage of providing a simple sensing method for Cys without any complicated synthetic operations being required for the incorporation of a functional ligand into the polymer backbone.

2. EXPERIMENTAL SECTION

2.1. Characterization. The NMR spectra were collected on a Bruker DRX-300 spectrometer with tetramethylsilane as the internal standard (Korea Basic Science Institute). Elemental analysis was performed on an Elemental Analyzer EA 1108 (Fisons Instruments). The UV−vis absorption spectra were recorded on a PerkinElmer Lambda 35 spectrometer. The photoluminescence spectra were obtained on a Varian Cary Eclipse fluorescence spectrophotometer equipped with a xenon flash lamp excitation source. Photographs of the fluorescent solution and solid were taken using a Canon Powershot A95 digital camera under a hand-held UV lamp. Dynamic light scattering (DLS) measurements were performed on a Photal ELS-Z2. Fluorescence image was performed on a Leica microscope DM5000B.

2.2. Synthesis. **1** was synthesized by a Suzuki cross-coupling reaction. 4,7-Bis(5-bromothiophen-2-yl)benzo-2,1,3-thiadiazole (26.1 mg, 0.057 mmol),[11d,12a] 1,4-dibromo-2,5-bis(4-sulfonatobutoxy)benzene sodium salt (300 mg, 0.514 mmol),[11d,12a] and 1,4-benzene diboronic acid bis(pinacol)-ester (188 mg, 0.571 mmol) were dissolved in a mixture of N,N-dimethylformamide (DMF, 8 mL), and an aqueous 2 M potassium carbonate solution (12 mL) in a round-bottom flask. After adding $(PPh_3)_4Pd(0)$ (3.5 mg, 0.003 mmol), the reaction mixture was stirred under argon at 100 °C for 48 h. After the reaction, the mixture was cooled and added slowly to a methanol/acetone/ether mixture (500 mL, 10: 40: 50, v/v/v). The precipitates were isolated by filtration. Finally, the polymer was purified by dialysis against water (Millipore Nanopure) using a 1.24 kD molecular weight cutoff cellulose membrane for 3 days. After dialysis, the polymer solution was freeze-dried to obtain a red solid (yield: 162 mg, 53%). ^{1}H NMR (300 MHz, D_2O, δ): 8.1−7.3 (br, 3.1H), 7.2−6.7 (br, 2H), 4.0 (br, 2.8H), 3.0 (br, 3H), 2.0−1.5 (br, 5H). ^{13}C NMR (75 MHz, D_2O, δ): 150.17, 130.56, 128.85, 128.26, 120.40, 117.63,114.98, 68.97, 65.76,51.21,49.86, 43.04, 27.93, 23.14, 21.30, 20.87. Anal. Calcd for $C_{20}H_{20.8}N_{0.2}S_{2.1}O_{7.2}Na_{1.8}$: C, 49.24; H, 4.27; N, 0.57; S, 13.79. Found: C, 48.48; H, 4.34; N, 0.50; S, 13.39.

2.3. Preparation of 1-Hg^{2+} -T complex. To the 5.44×10^{-6} M of **1** was dissolved in 0.1 M sodium phosphate buffer solution (pH 7.4). Then, to this solution was added 2.1×10^{-3} M of thymine. To induce the interaction with sulfur in **1** and Hg^{2+}, the solution was mixed with 6.0×10^{-4} M of Hg^{2+} and stirred at room temperature. The polymer−mercury−thymine complex was formed via specific binding of Hg^{2+}-T and Hg^{2+}-sulfur atom in the polymer.

2.4. Fluorescence Detection for Cys. To **1**-Hg^{2+}-T complex were added a various concentrations of Cys and stirred in 0.1 M sodium phosphate buffer solution (pH 7.4). To investigate the fluorescence color change of the complex in the presence of Cys, we measured the photoluminescence spectra at excitation wavelength of 350 nm.

2.5. DLS Measurement upon Addition of Cys. To the **1**-Hg^{2+}-T complex was added each concentration of Cys solution; we stirred in 0.1 M sodium phosphate buffer solution (pH 7.4) and measured the size of aggregates using dynamic light scattering method.

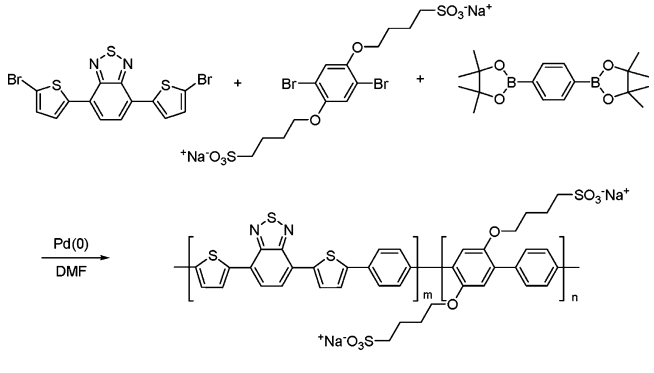
2.6. Zebrafish Maintenance. Zebrafishes were raised and kept under standard laboratory conditions at 28.5 °C. Zebrafish embryos were obtained from natural spawnings. Embryos were staged at specific hours post fertilization (hpf).

2.7. Fluorescence Imaging of Zebrafish. The 55 hpf zebrafish embryos were incubated with mercury ion (2.0×10^{-7} M) for 40 min in egg water. After Hg^{2+} treatment, embryos were washed for 30 min and **1** was microinjected into the sinus venosa/cardinal vein. After washing for 30 min with egg water, mercury and **1**-treated zebrafish embryos were finally incubated with Cys solution (4.0×10^{-7} M) for 2 h and washed with egg water.

3. RESULTS AND DISCUSSION

Conjugated polyelectrolyte **1** was synthesized with a good yield from the corresponding monomers. The detailed Suzuki coupling polymerization procedure is described in Scheme 1.[11d,12a] The molar composition of the polymer, x and y

Scheme 1. Polymerization of Water-Soluble Conjugated Polymer 1



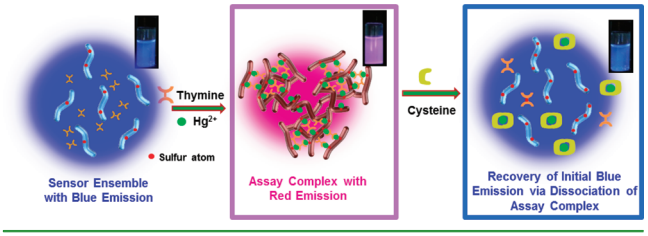
1

was optimized in terms of the trade-off between the water-solubility (n) and exciton migration effect (m). Elemental analysis revealed that the composition of **1** was 0.07: 0.93 (m:n) in terms of the mole fraction. To determine the molecular weight of **1**, we carried out gel permeation chromatography (GPC) to using an aqueous mobile phase, but in that case the adsorption of polymer to the columns occurred. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) was also attempted, but the results were not satisfied to get feasible data.[11d,14]

As mentioned above, **1** showed unique optical properties such as a change in its emission maximum depending on its phase. In aqueous solution, **1** exhibited a blue emission at 420 nm, while in the solid state it showed red emission at 653 nm (see Figure S1 in the Supporting Information), caused by the well-known phenomenon of intermolecular exciton migration. To develop a sensing strategy, an assay complex was prepared by simply mixing suitable amounts of **1**, T, and Hg^{2+} to induce aggregation with a red emission. By adding Hg^{2+}, the fluorescence intensity at short wavelength (420 nm) of the sensor ensemble (**1**+T) decreased and concomitantly a new emission at long wavelength (653 nm) appeared and the intensity increased (see Figure S2a in the Supporting Information). This occurred because of the self-aggregation of the mixture, which was induced by the intermolecular interaction among **1**, T, and Hg^{2+}. It is known that Hg^{2+} can interact with T to form a T-Hg^{2+}-T complex[12b−d] as well as with sulfur-containing species.[12e,f] Hg^{2+}- and T-mediated intermolecular $\pi-\pi$ stacking induces polymer aggregation leading to fluorescence enhancement or quenching to detect mercury ions. **1** could be simply quenched when exposed to Hg^{2+} in the abscence of T, mainly due to the heavy atom effect (see Figure S2b in the Supporting Information),[12g] implying an interaction between Hg^{2+} and **1** is not enough to induce molecular aggregation. In the simultaneous presence of Hg^{2+} and T, the polymer readily formed intermolecular aggregates through the polymer-Hg^{2+}-T complex via a cooperative intermolecular linkage.[12a]

On the basis of the formation of a long wavelength emission upon aggregation, we demonstrated a new system to detect Cys via the recovery of the short wavelength emission. The Cys detection assay is illustrated in Scheme 2. First, to prepare the

Scheme 2. Working Principle of Assay Complex As a Sensor System in Response to Cysteine



sensor assay complex, the blue-emitting anionic polyelectrolyte **1** was treated with T, and then combined with the Hg^{2+} in the aqueous phase. At this stage, an aggregation-induced strong red emission could be observed. Upon the addition of Cys to the assay, on the contrary, the aggregates disappeared instantaneously and the original blue emission was restored. This implies that Hg^{2+}, which was an important building block of **1**-Hg^{2+}-T, left the **1**-Hg^{2+}-T assay complex to form a more favorable interaction with Cys.[5e]

Cys has a strong interaction with Hg^{2+} because of the thiol group when it is added to the assay. Thus Cys has stronger interaction with Hg^{2+}, extracts Hg^{2+} from the **1**-Hg^{2+}-T assay complex, and thereby, the assay complex became dissociated into individual molecules, resulting in the restoration of the blue emission. On the basis of the preferred Hg^{2+}-Cys interaction, the emission changes of the **1**-Hg^{2+}-T assay with different concentrations of Cys in 0.1 M sodium phosphate buffer solution at pH 7.4 were investigated as shown in Figure. 1a.

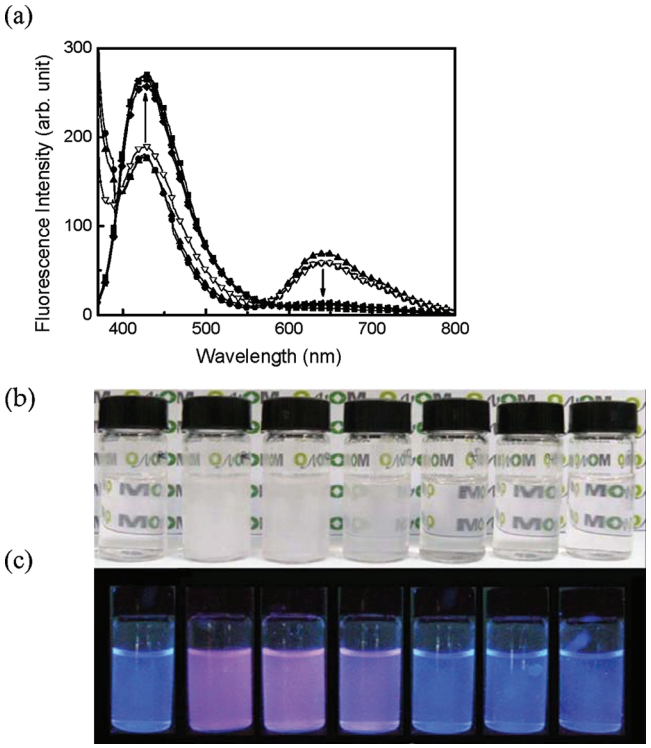


Figure 1. (a) Changes in the fluorescence spectra of polymer **1**-Hg^{2+}-T complexes ([**1**] = 5.44×10^{-6} M, [Hg^{2+}] = 1.20×10^{-3} M, [T] = 6.30×10^{-3} M) in 0.1 M sodium phosphate buffer solution (pH 7.4) at different concentrations of Cys (■, **1**; ●, **1**-Hg^{2+}-T; ▲, **1**-Hg^{2+}-T + Cys [2.50×10^{-4} M]; ▽, **1**-Hg^{2+}-T + Cys [5.00×10^{-4} M]; ♦, **1**-Hg^{2+}-T + Cys [7.50×10^{-4} M]; ◀, **1**-Hg^{2+}-T + Cys [1.00×10^{-3} M]; right-facing unfilled triangle, **1**-Hg^{2+}-T + Cys [1.50×10^{-3} M]. The fluorescence spectra were obtained using an excitation wavelength λ_{ex} = 350 nm. Photographs of **1**-Hg^{2+}-T assay complex in 0.1 M sodium phosphate buffer solution (pH 7.4) at different concentrations of Cys (from left to right: **1**, **1**-Hg^{2+}-T + Cys where [Cys] = 0, 2.50×10^{-4}, 5.00×10^{-4}, 7.50×10^{-4}, 1.00×10^{-3}, 1.25×10^{-3} M) (b) under ambient light and (c) UV light with a wavelength of 365 nm.

With increasing Cys concentration, the red emission at 653 nm clearly decreased in intensity and, at the same time, the intensity of the blue emission at 420 nm recovered to that of the initial **1**-T solution. The limit of detection (LOD) was estimated according to the published method[5b,e] and determined to be 6.0×10^{-5} M.

The fluorescence spectra clearly show that the dissociation-induced recovery of the emission intensity at 420 nm reaches the initial fluorescence feature of the aqueous polymer solution and, accordingly, a red-to-blue emission color change takes place (Figure 1a). The assay complex shows not only a change in the emission color from red-to-blue (Figure 1c) but also a change in the solubility (opaque dispersion-to-transparent solution) in the buffer solution upon its exposure to Cys (Figure 1b).

To gain insight about the aggregation and dissociation of the assay complex upon the addition of Cys, dynamic light scattering (DLS) measurements were performed as shown in Figure S3 in the Supporting Information. For the sample without any Cys in the assay complex (**1**-Hg^{2+}-T), the particle size was measured to be approximately 2000 nm and the complex solution was opaque due to the presence of the aggregates. After the addition of Cys of 2.50×10^{-4} M, the particle size decreased to 1396 nm. The further addition of Cys led to a rapid decrease of the particle size to about zero and, thus, the solution became clearly transparent. According to the DLS data, it can be concluded that the particle size of the complex gradually decreased upon the addition of Cys, showing a similar trend to the decrease in the emission intensity at 653 nm. All of these aforementioned results indicate that Cys plays an important role in the dissociation of the assay complex, thereby providing a decrease in its particle size and fluorescence color change of red-to-blue.

To determine the selectivity of this assay complex, we investigated its fluorescence response to other amino acids. In Figure 2a, the fluorescence color of the assay complex did not change in the presence of the other amino acids. It is likely that these other amino acids do not have any interaction with Hg^{2+}, because significant aggregation-induced emission color changes of the assay complex were not observed. The results clearly indicate that this system demonstrates high selectivity to Cys as shown in Figure 2b. The emission color and opaque dispersion of the aggregates in the presence of all of the amino acids except Cys remained unchanged at the same concentration of Cys (Figure 2c).

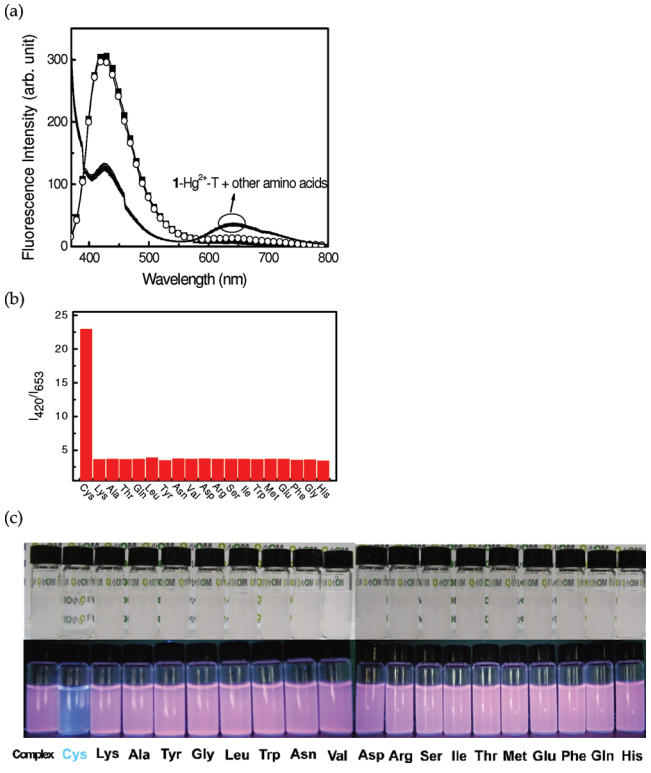


Figure 2. (a) Fluorescence spectra of the **1**-Hg^{2+}-T assay complex with various amino acids ([**1**] = 5.44×10^{-6} M, [amino acid] = 1.20×10^{-3} M, [T] = 6.30×10^{-3} M), (■) **1**, (○) **1**-Hg^{2+}-T + Cys. (b) Relative changes in fluorescence intensity at 420 and 653 nm of **1**-Hg^{2+}-T assay complex in the presence of various amino acids in 0.1 M sodium phosphate buffer solution (pH 7.4). The fluorescence spectra were obtained using an excitation wavelength λ_{ex} = 350 nm. (c) Photographs of the **1**-Hg^{2+}-T assay complex in the presence and absence of amino acids under ambient light (upper) and UV light (bottom).

Notably, methionine (Met), which contains sulfur in the form of the thioester group, did not affect the changes in the emission color. Moreover, amino acids that have potential N-type ligands as basic amino groups, such as histidine (His) and lysine (Lys), did not show sufficient interaction with the Hg^{2+} to induce a color change. Other sulfur-containing amino acids such as homocysteine (Hcy) and glutathione (GSH) show slight emission color change (see Figure S4 in the Supporting Information). The changes in intensity were not intense compared to the case of Cys and this phenomenon has been observed in the previous studies.[6k,15] The assay complex (**1**-Hg^{2+}-T) in the buffer solution was stable for a month without significant changes in emission intensity or emission shift (see Figure S5 in the Supporting Information).

We evaluated the behavior of **1** with Hg^{2+} and Cys for the fluorescence monitoring of a vertebrate organism, the zebrafish, and the schematic procedure is illustrated in Figure S6 in the Supporting Information. The zebrafish were incubated with Hg^{2+} for 40 min, followed by the microinjection of **1** into the heart of the 55 hpf zebrafish embryos. Then, the zebrafish embryos were treated with Cys solution. The photographs obtained by fluorescence microscopy demonstrate clearly that the location of Hg^{2+} in the zebrafish embryos as shown in Figure 3. The zebrafish microinjected with **1** exhibited a very weak red fluorescence color (Figure 3a). However, as shown in Figure 3b, when the zebrafish were exposed to the Hg^{2+} prior to their injection with **1**, a stronger red emission was observed compared to the case of Figure 3a. In this case, the blood vessels of the zebrafish emitted a red fluorescence color. This seems to result from the intensified aggregation of **1**, because the accumulated Hg^{2+} induced the aggregation of the polymer when the zebrafish were pretreated with Hg^{2+}. Finally, when the Hg^{2+} and **1**-treated zebrafish embryos were incubated with Cys, the fluorescence intensity was diminished, presumably due to the removal of the Hg^{2+} by Cys (Figure 3c). These in vivo

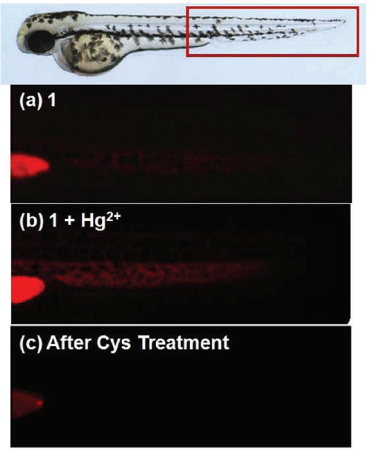


Figure 3. Phase contrast image and fluorescence image of zebrafish. Fluorescence image of (a) zebrafish injected with **1** only; (b) zebrafish injected with **1** after incubation with Hg^{2+}; (c) zebrafish treated with Cys after incubation of $Hg^{2+}$and injection of **1** ([**1**] = 5.0×10^{-5} M, [Hg^{2+}] = 2.0×10^{-7} M, [Cys] = 6.0×10^{-7} M).

studies demonstrate that this mechanism is able to detect Cys in a biological environment.

CONCLUSIONS

In conclusion, we developed a highly selective assay complex comprising the **1**-Hg^{2+}-T complex for detecting Cys at micromolar concentrations in water. The complex of **1**-Hg^{2+}-T was prepared through the specific binding of **1**, Hg^{2+}, and T and exhibited a fluorescence color change (blue-to-red) by the formation of complex aggregates. This attractive complex was possible to use as a highly selective sensor which had an effective emission color change (red-to-blue) upon exposure to Cys, resulting from the stronger interaction between Hg^{2+} and Cys which made **1** highly soluble in water. The fluorescence color change is selective for Cys compared to other amino acids. This system has the advantage of having a facile method of preparation without functional groups for the detection of Cys. Finally, the in vivo study clearly demonstrated that **1** is a potential biosensory platform in a biological environment.

ASSOCIATED CONTENT

Supporting Information

Experimental details; PL spectra and DLS measurement of the resulting samples. 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 interest.

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