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A Sensitive and Selective Near-Infrared Fluorescent Probe for Mercuric Ions and Its Biological Imaging Applications

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A new mercury(II) near-infrared region fluorescent probe 3,9-dithia-6-monoazaundecane-tricarbocyanine has been designed and synthesized. It consists of two functional moieties: the tricarbocyanine performs as the near-infrared region fluorophore, and the 3,9-dithia-6-monoazaundecane acts as the selected binding site for metal ions. The near-IR excitation and emission profiles of the probe can minimize cell and tissue damage and avoid native fluorescence from natural cellular species. It exhibits fluorescence increase upon the binding of the Hg^{2+} based on the inhibition of the photoinduced electron transfer quenching mechanism. Excellent sensitivity and selectivity for mercuric ions are observed with this probe. The value of the system is demonstrated by its use in monitoring the real-time uptake of Hg^{2+} within HepG2 cells and five day old zebrafish. The synthesis and remarkable properties of it help to extend the development of metal ions fluorescent probes for biological applications.

Introduction

Mercury is one of the most toxic heavy metals on earth.[1–3] Mercury contamination is widespread and an estimated total of 4400 to 7500 metric tons of mercury is released into the environment worldwide annually.[4] It exists in a variety of different forms (metallic, ionic, and as part of organic and inorganic salts and complexes).[5] Solvated inorganic mercuric ion (Hg^{2+}), one of the most stable inorganic forms of mercury, is a caustic and carcinogenic material with high cellular toxicity[6–8] and can cause damage to the brain, heart, kidney, stomach, intestine, and many other organs.[9] In addition, the bacteria in aqueous environments can transform inorganic mercury into methylmercury, which can accumulate in seafood and then enter the food chain,[10–14] once absorbed into the human body, methylmercury results in several serious disorders including sensory, endocrine, and neurological damage[15] with acute and chronic toxicity. Therefore, concerns over toxic mercury have motivated us to develop highly selective and sensitive methods to monitor mercury, it is especially useful in making mercury visible in living cells and particularly organisms.

Current methods for detecting mercuric ions, such as atomic absorption/emission spectroscopy[16] and inductively coupled plasma mass spectrometry,[17,18] display high sensitivity and selectivity and are usually used for quantitive analysis. However, these assays are not suitable for monitoring mercury in vivo. Compared with the above techniques, the fluorescent method is a powerful tool in cell biology because of its generally nondestructive character and high sensitivity and specificity. Moreover, fluorescence microscopic imaging techniques can map the temporal and spatial distribution of mercuric ions within living cells. Recently, many fluorescent probes for Hg^{2+} detection have been developed (that is, using small organic molecules,[19–25] DNAzymes,[26,27] protein,[28,29] gold nanoparticles,[30–32] and oligodeoxyribonucleotide-based probes[33]), but only few Hg^{2+} imaging studies have been reported.[34–36] Liu[34] and Chang[35] applied the mercury sensor to cultured HEK cells separately, and Shin et al.[36] presented a rhodamine-based probe and obtained fluorescence images in living cells and zebrafish.

Near-infrared region (NIR) around 650–900 nm, where only a few classes of molecules exhibit absorption, and they do not contribute to the fluorescence signal, is an area of low background fluorescence interference in biological systems.[37] The feature of the particular spectral region make the NIR probe more suitable for intracellular fluorescent imaging. In addition, near-infrared light can penetrate more deeply into tissues, which is of great importance to study living organism imaging. For example, Nagano et al. reported two NIR probes used to monitor zinc[38] and NO[39] respectively, and previously, we developed a NIR probe for detecting zinc in macrophage cells.[40] However, there are only a few NIR fluorescent probes for imaging mercuric ions. Wong et al.[41] have reported a NIR probe for mercuric ions based on fluorescence quenching, however this probe cannot be applied in cells to visualize Hg^{2+}. Thus, it is appealing to develop NIR probes to detect Hg^{2+} in living cells, and particularly, organisms.

Herein, we reported the synthesis and properties of a novel NIR probe DMA-Cy for imaging Hg^{2+}, based on a photoinduced electron transfer (PET) quenching mechanism.[42–44] We selected tricarbocyanine with two propyl groups as the NIR fluorophore. The near-IR excitation and emission profiles of tri-

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carbocyanine can limit photodamage to biological samples and avoid autofluorescence from native cellular species. 3,9-dithia-6-monoazaundecane (DMA) is chosen as the metal receptor[25,45–47] because of its high selectivity toward Hg^{2+}. We synthesized DMA-Cy and characterized it by ^{1}H NMR, ^{13}C NMR, and mass spectrometry. The probe can respond fast to mercuric ions in aqueous solution. Job plot analysis reveals that DMA-Cy forms a 1:1 stoichiometry complex with mercury. Additionally, the probe displays a high selectivity for Hg^{2+}. Based on its ideal chemical and spectroscopic properties, we have demonstrated the value of the probe in HepG2 cells and five day old zebrafish by confocal microscopy.

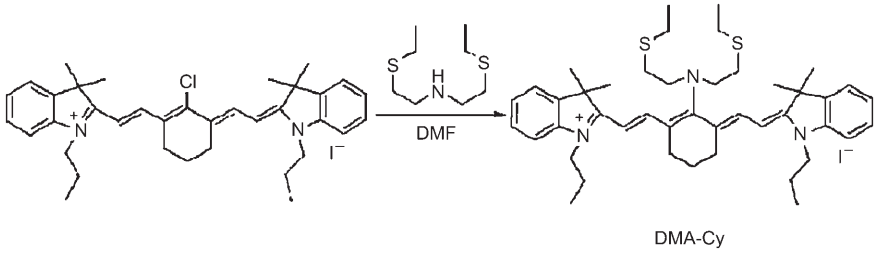
Results and Discussion

Design and synthesis of DMA-Cy

Our strategy for designing the probe 3,9-dithia-6-monoazaundecane-tricarbocyanine (DMA-Cy) is based on a photoinduced electron transfer (PET) quenching mechanism. It consists of two separate functional moieties: the tricarbocyanine with two propyl groups performs as the NIR fluorophore, which had been widely used in various fields and employed as fluorescent labels in fluorescence imaging studies of biological mechanisms because of its high quantum yield, large stokes shift, and good biological compatibility, and the 3,9-dithia-6-monoazaundecane acts as the selective binding site for metal ions because of its high selectivity toward Hg^{2+}. The vinyl chlorine on the cyclohexane bridgehead of tricarbocyanine is reactive and can be replaced by DMA, so the probe can be easily synthesized with a one-step reaction under mild conditions (Scheme 1).

Spectral properties of DMA-Cy

DMA-Cy was evaluated under buffered aqueous acetonitrile solution (10 mM PBS, pH 7.40, 1% acetonitrile as co-solvent). The fluorescence spectrum of DMA-Cy showed that λ_{max} of excitation and emission lie at 685 and 763 nm, respectively (Figure 1), and as expected, the fluorescence intensity was very low. The fluorescence quantum yield was 0.02, which was determined in methanol in reference to rhodamine B ($\Phi_f = 0.69$ in methanol).[40] The low quantum yield of the unbound probe resulted from PET quenching of the tricarbocyanine emission by the lone pair of the nitrogen atom. In the presence of Hg^{2+}, because of the inhibition of PET by co-ordination of mercury,



Scheme 1. Synthesis of DMA-Cy.

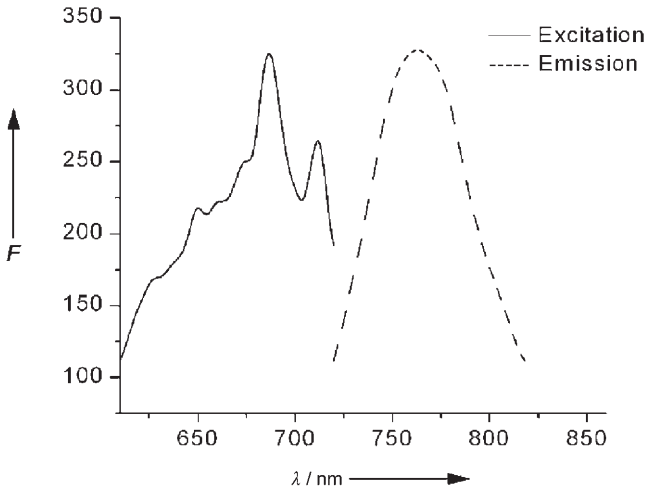


Figure 1. Fluorescence spectra of DMA-Cy (2.00 μM).

strong fluorescence was obtained immediately and there was no evident change in the excitation and emission wavelength (Figure 2). The fluorescence quantum yield increased to 0.12.

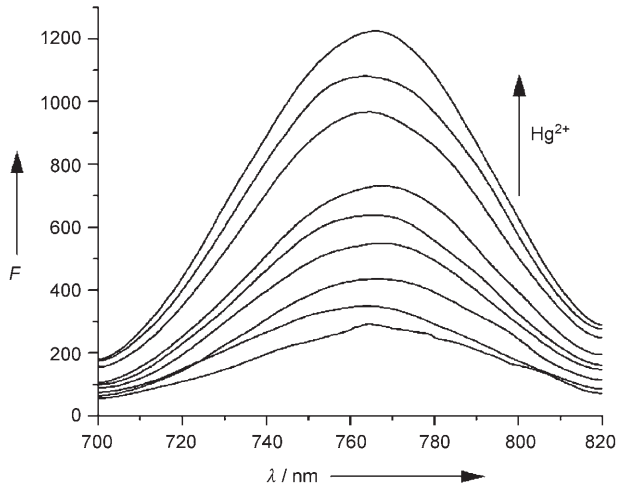


Figure 2. Fluorescence responses of DMA-Cy (2.00 μM) to different concentrations of Hg^{2+} (0, 0.10, 0.20, 0.50, 0.80, 1.10, 1.40, 1.70, and 2.00 μM respectively) in buffered aqueous acetonitrile solution (10 mM PBS, pH 7.40). Spectra were obtained with excitation at 685 nm.

To determine the stoichiometry of the DMA–Cy/Hg^{2+} complex, Job's method was applied, keeping the sum of the initial concentration of DMA-Cy and Hg^{2+} at 4.00 μM, and changing the molar ratio of $[Hg^{2+}]/([Hg^{2+}]+[\text{DMA-Cy}])$ from 0 to 1. As shown in Figure 3, the inflection point was at 0.5, thus demonstrating that DMA-Cy formed a 1:1 stoichiometry complex with mercury in solution. This is consistent with the spectral properties of DMA-Cy that the

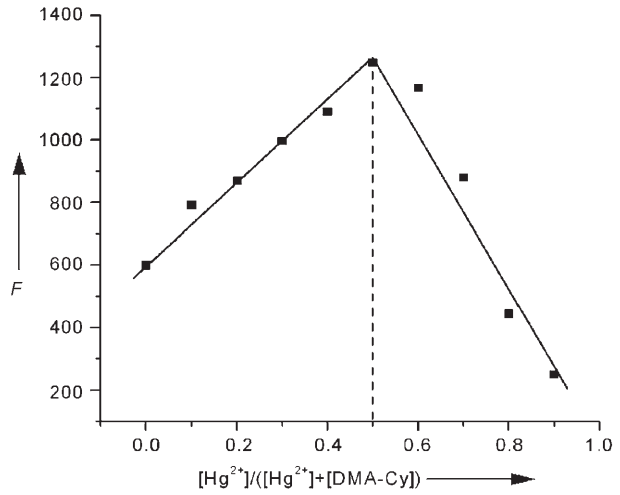


Figure 3. Job plot of the complexation between DMA-Cy and Hg^{2+}.

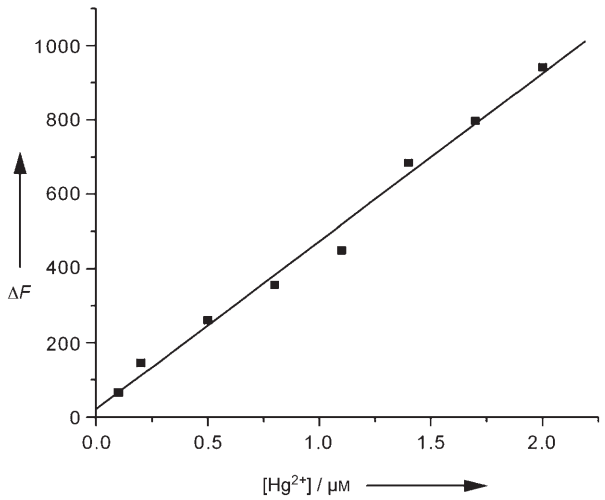


Figure 5. A linear correlation between the relative fluorescence intensity and the concentrations of Hg^{2+}.

fluorescence intensity increased greatly upon increasing Hg^{2+} and the increase stopped after 1 equiv of Hg^{2+}.

In the experiment, the pH of the medium has a great effect on fluorescence intensity. One important observation was that the relative fluorescence intensity was high and independent of pH in the range of 7.00 to 7.80 (Figure 4), so pH 7.40 of

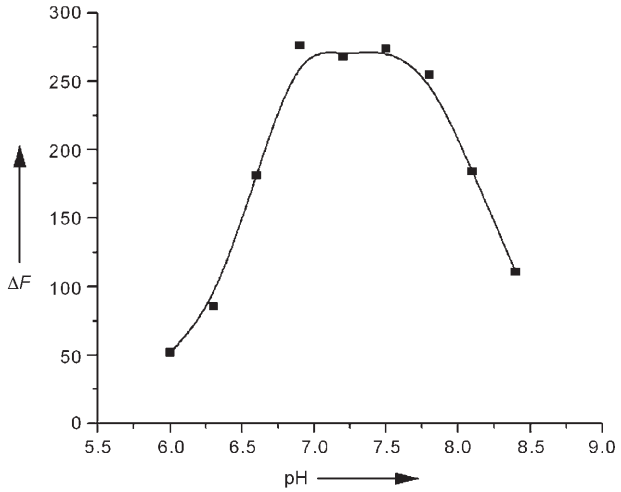


Figure 4. Effect of pH values. The concentration of DMA-Cy was 2.00 μM, Hg^{2+} was 0.50 μM.

simulated physiological conditions was selected. Besides, KH_2PO_4-Na_2HPO_4 (pH 7.40) is a sensitive buffer system and has often been used as buffer solution in biological systems, thus, this phosphate buffer solution (10 mM) was chosen in our experiments.

The fluorescence intensity was directly proportional to the amount of Hg^{2+}. There was a good linearity between relative fluorescence intensity and concentration of Hg^{2+} in the range of 1.00×10^{-7} M to 2.00×10^{-6} M with 2.00 μM of DMA-Cy. The regression equation was $(F-F_0)=21.75+451.92\times[Hg^{2+}]$ ($\times10^{-6}$ M) with a linear coefficient of 0.9943 (Figure 5), and the limit of detection (LOD) of the proposed method was 1.39×10^{-8} M. This result demonstrated that the probe could detect Hg^{2+} both qualitatively and quantitatively. The apparent dissociation constant K_d was determined to be 7.90×10^{-7} M.

The ability of the probe to select biologically relevant metal ions is an important requirement for biological applications. To assess the selectivity of the method, the effect of other metal ions was examined individually. An error of ±5.0% in the relative fluorescence intensity was considered tolerable. The fluorescence response of DMA-Cy (2.00 μM) to the presence of various metal ions and its selectivity for Hg^{2+} are shown in Figure 6. K^+, Na^+, Ca^{2+}, and Mg^{2+}, which exist at high levels in living cells, did not interfere even at high concentration. Besides, DMA-Cy was also selective for Hg^{2+} over Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+} , Zn^{2+}, Cd^{2+} , Pb^{2+}, and Ag^+. This indicated that

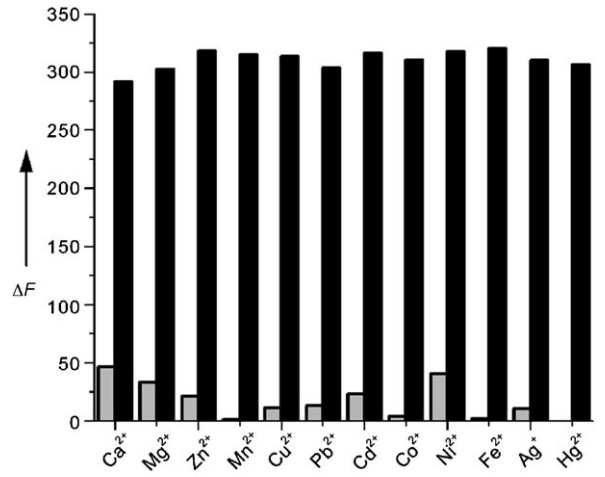


Figure 6. The relative fluorescence intensity of DMA-Cy (2.00 μM) in pH 7.40 (10 mM PBS) to various metal ions. Light gray bars represent the addition of the analytes: Ca^{2+} (1.5 mM); Mg^{2+} (0.25 mM); Zn^{2+} (25 μM); Mn^{2+} (50 μM); Cu^{2+} (25 μM); Pb^{2+} (50 μM); Cd^{2+} (25 μM); Co^{2+} (50 μM); Ni^{2+} (50 μM); Fe^{2+} (50 μM); Ag^+ (25 μM). Black bars represent the subsequent addition of 0.50 μM Hg^{2+} to the solution.

the DMA-Cy was highly selective for Hg^{2+} over competing metal ion analytes in aqueous solution because of its thioether-rich receptor.

Confocal fluorescence imaging experiments

In vivo detection of Hg^{2+} in cells: Because of its ideal chemical and spectroscopic properties, DMA-Cy should be suitable to detecting mercuric ions in living cells and organisms. To test this proposal, we first applied the probe to HepG2 (human cancerous liver cells) using confocal microscopy (Figure 7).

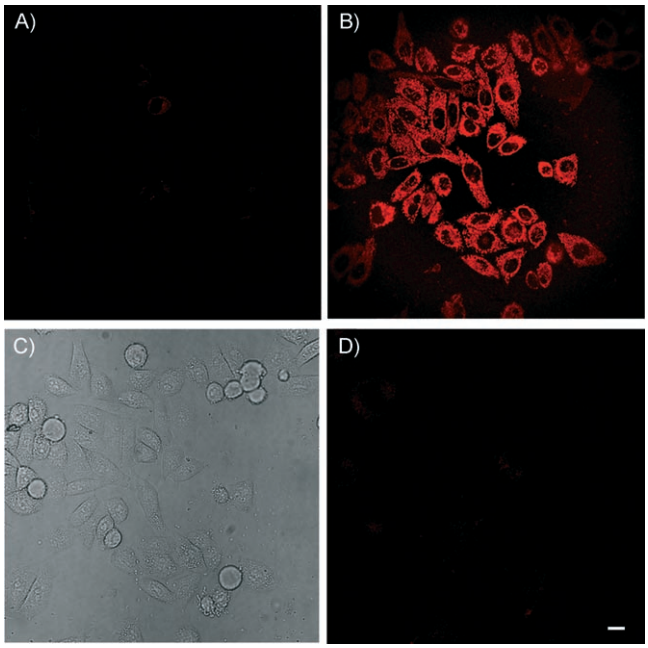


Figure 7. Confocal fluorescence images of Hg^{2+} in live HepG2 cells (Leica confocal fluorescence microscope, 40× objective lens). A) fluorescence image of HepG2 cells incubated with DMA-Cy. B) HepG2 cells incubated with DMA-Cy, washed three times, and then further incubated with Hg^{2+}. C) bright field image of HepG2 cells shown in panel B). D) DMA-Cy-supplemented cells pretreated with Hg^{2+}, then treatment with TPEN. Scale bar = 10 μm.

HepG2 incubated with DMA-Cy (2.00 μM) for 20 min at 37 °C showed negligible fluorescence (Figure 7A). In contrast, DMA-Cy-stained cells displayed fluorescence by the addition of $HgCl_2$ (2.00 μM) into the medium and incubation for another 20 min at 37 °C (Figure 7B), and brightfield image measurement confirmed that the cells were viable throughout the imaging studies (Figure 7C). On treatment of the cells with 1 equiv of the high affinity mercuric chelator, *N,N,N′,N′*-tetra (2-picolyl) ethylenediamine (TPEN) for 20 min at 37 °C, the fluorescence decreased to baseline (Figure 7D). This on-off behavior could be reversed by addition of another 1 equiv of Hg^{2+}. Control experiments demonstrated the probe bound to mercuric ions reversibly.

In vivo detection of Hg^{2+} in zebrafish: The success obtained within the cell experiments encouraged us to determine if the probe could be used to detect Hg^{2+} in living organisms, so in vivo detection in zebrafish was evaluated. Five day old zebrafish were first incubated with DMA-Cy (10.00 μM) in sea salt water for 20 min at 28 °C, and then followed by addition of 1 equiv of $HgCl_2$ for another 20 min at 28 °C after removal of the remaining probe, the fluorescence images were acquired (Figure 8). Taken together, the results of the confocal fluorescence microscope analysis showed that the probe should be potentially useful for the study of the toxicity or bioactivity of Hg^{2+} in living organisms.

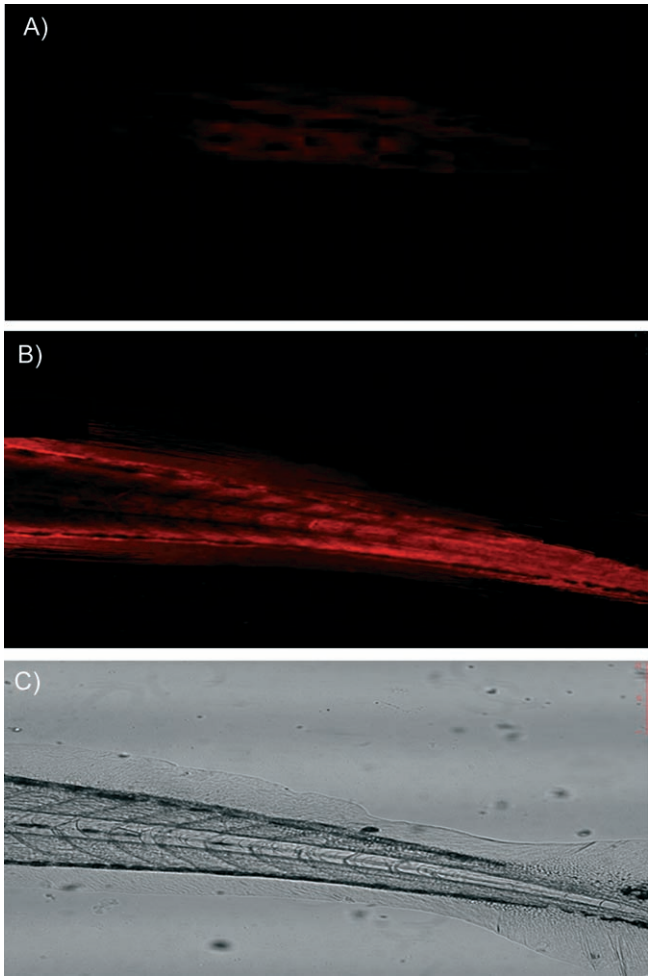


Figure 8. Confocal fluorescence images of Hg^{2+} in five day old zebrafish (10× objective lens). Imaging was carried out after washing the zebrafish with seawater (3×). A) Fluorescence image of five day old zebrafish incubated with DMA-Cy (10.00 μM). B) Fluorescence image of five day old zebrafish incubated with DMA-Cy, washed three times, and then further incubated with Hg^{2+}. C) Bright field image of five day old zebrafish shown in (B).

Conclusions

In summary, we have described the chemical synthesis, property analysis, and biological applications of 3,9-dithia-6-monoazaundecane-tricarbocyanine, which is a new type of tricarbocyanine-based near-infrared region fluorescent probe for imaging mercuric ions in biological systems. The near-IR excitation and emission profiles of the probe can minimize the cell and

tissue damage and avoid native fluorescence from natural cellular species. This probe responds to mercuric ions stoichiometrically, rapidly, and reversibly at room temperature and produces the strongly fluorescent product based on a photoinduced electron transfer (PET) quenching mechanism. Excellent sensitivity and selectivity for mercuric ions are observed with the probe. The value of the probe is demonstrated by its use in monitoring the real-time uptake of mercuric ions in HepG2 cells and five day old zebrafish. So this system can be employed to detect Hg^{2+} in biological samples. The synthesis and remarkable properties of it help to extend the development of metal ions fluorescent sensors for biological applications.

Experimental Section

Synthetic materials and methods: Ethanethiol was purchased from Sigma–Aldrich. Tricarbocyanine with two propyl groups was synthesized in our laboratory. All other reagents and solvents were purchased from commercial sources and of the highest grade. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Brucker Avance 300 MHz. Mass spectral determination was made on an Agilent HP 1100 LC-MSD (U.S). Melting points were measured on a Yanaco micro-melting point apparatus (Yanagimoto MFG Co., Kyoto, Japan), Silica gel (100–200 mesh, Qingdao Haiyang Chemical Co.) was used for column chromatography. Analytical thin-layer chromatography (TLC) was performed using GF254 silica gel (precoated sheets, 0.77 mm thick, Taizhou Si-Jia Biochemical Plastic Company).

Synthesis of 3,9-dithia-6-azaundecane (DMA): The compound was synthesized according to the literature[11] in a yield of 80% and identified by ^{1}H NMR ($CDCl_3$ 300 MHz): $\delta = 1.22$ (t, 6H), 1.88 (s,1H), 2.52 (m, 4H), 2.70 (t, 4H), 2.81 (t, 4H).

Synthesis of 3,9-dithia-6-monoazaundecane-tricarbocyanine (DMA-Cy): Tricarbocyanine (333 mg, 0.50 mmol) was dissolved in anhydrous DMF (15 mL) in a round bottom flask, and then 3,9-dithia-6-monoazaundecane (965 mg, 5.0 mmol) was added dropwise. The mixture was stirred at 68–70 °C under argon for 4 h with progress monitored by TLC, followed by cooling to room temperature and added into ether (300 mL) with violent stirring. The vinyl chlorine on the cyclohexane bridgehead of tricarbocyanine was reactive and could be replaced by DMA, so the probe was easily synthesized through a one-step reaction under mild conditions. The obtained blue solid was filtered and dried under vacuum, then purified on silica gel chromatography eluted with ethyl acetate/methanol (4:1, 123 mg, 30%). m.p.233.5–235 °C; ^{1}H NMR of DMA-Cy ($CDCl_3$ 300 MHz): $\delta = 0.88$–0.96 (t, 6H), 1.26 (t,6H), 1.56–1.69 (t, 4H), 1.87 (t, 2H), 2.08 (d, 12H), 2.55 (m, 4H), 2.75 (t, 4H), 2.88–2.96 (m, 8H), 3.64–3.92 (t, 4H), 5.85–5.90 (d, 2H), 7.02–7.53 (m, 8H), 8.02 (d, 2H); ^{13}C NMR of DMA-Cy ($CDCl_3$ 300 MHz): $\delta = 11.6$, 14.7, 14.9, 22.4, 25.8, 25.9, 26.0, 26.1, 26.2, 28.7, 28.9, 29.6, 29.8, 41.6, 42.7, 47.4, 47.9, 48.3, 53.9, 54.2, 57.1, 109.6, 109.7, 122.0, 124.0, 124.3, 128.5, 128.6, 128.7, 140.2, 140.3, 142.6, 142.8, 162.9, 175.9; ESI-MS calcd for $= C_{44}H_{62}N_3S_2$: 696.0 $[M-I]^-$; found 696.4.

Spectroscopic materials and methods: Ultrapure water (18.2 MΩ cm) was purified by using an arium 611VF system with ultrafilter and UV lamp (Sartorius, Germany). The fluorescence spectra and fluorescence intensity were measured on FLS-920 Edinburgh fluorescence spectrometer with a Xenon lamp and 1.0 cm quartz cells at the slits of 2.5/2.5 nm. All pH measurements were made with a pH-3c digital pH-meter (Shanghai Lei Ci Device Works, Shanghai, China) with a combined glass-calomel electrode. HepG2 cells were purchased from the committee on type culture collection of Chinese academy of sciences. Dulbecco's modified Eagle's medium (DMEM) were purchased from Sigma. The images were taken using a LTE confocal laser scanning microscope (Germany Leica Co., Ltd) with objective lenses (×40, ×10) and the excitation wavelength was 635 nm.

Sample preparation: A stock solution (200.00 μM) of DMA-Cy was prepared by dissolving in acetonitrile. Probe solutions (20.00 μM) were prepared by diluting a stock solution of DMA-Cy with ultrapure water. Solutions of $HgCl_2$ (20.00 μM), PBS (0.10 M) were prepared with ultrapure water.

Fluorescence analysis: Into a 10 mL color comparison tube were added solutions of DMA-Cy (20.00 μM, 1.00 mL), different concentrations of $HgCl_2$, and PBS solution (0.10 M, 1.00 mL, pH 7.40) in turn. After diluted to 10.00 mL volume with ultrapure water, the mixture was equilibrated, and the fluorescence intensity was measured at $\lambda_{ex}/\lambda_{em} = 685/763$ nm against a reagent blank at the same time. The excitation and emission slits were set to 2.5 nm, respectively.

Staining of cell cultures and confocal fluorescence imaging: HepG2 cells (human hepatocellular liver carcinoma cell line) were maintained following protocols provided by the American type tissue culture collection. Cells were seeded at a density of 10^5 cells ml^{-1} in high glucose Dulbecco's Modified Eagle Medium (DMEM, 4.5 g of glucose per L) supplemented with 10% fetal bovine serum (FBS), $NaHCO_3$ (2 g L^{-1}) and 1% antibiotics (penicillin/streptomycin, 100 U mL^{-1}). Cultures were maintained at 37 °C under a humidified atmosphere containing 5% CO_2. Cell imaging was carried out after washing cells with PBS solution (pH 7.40) for three times.

Zebrafish were breed at optimal conditions in sea salt water and kept at 28 °C. The five-day-old zebrafish were incubated with DMA-Cy (10.00 μM) for 20 min at 28 °C. After washing with sea salt water to remove the remaining sensor, the zebrafish were further incubated with $HgCl_2$ (10.00 μM) for another 20 min at 28 °C. Before imaging, the fish were washed with sea salt water.

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