A fluorescein derivative FLTC as a chemosensor for Hg^{2+} and Ag^+ and its application in living-cell imaging

Wei Shen,^{a*} Lin Wang,^b Min Wu^c and Xiaofeng Bao^{d*}

Abstract: FLTC was synthesized and used as a fluorescent chemosensor to detect Hg^{2+} . It showed high selectivity toward Hg^{2+} over many heavy metal ions in an ethanol- H_2O (3:2, v/v, HEPES buffer, 0.5mM, pH 7.15) solution with a detection limit of 0.21 μM . After complexation with Hg^{2+} , FLTC showed extremely high selectivity toward Ag^+ with a detection limit of 0.009 μM . Therefore, detection of Hg^{2+} and Ag^+ could be realized using FLTC and the FLTC- Hg^{2+} complex, respectively. Cytotoxicity assays and fluorescence microscopy analysis showed that FLTC could be used as a fluorescent probe to detect Hg^{2+} and Ag^+ in L-02 human liver cells.

Key words: fluorescein derivative; chemosensor, Hg^{2+} , Ag^+ , living cell imaging

a Jiangsu Key Laboratory of Pesticide Science, Department of Science, Nanjing Agricultural university, 1 tongwei road, Nanjing, 210095, P.R. China swqdj@njau.edu.cn

b School of Biology and Chemical Engineering, Jiangsu University of Science and Technology, Zhenjiang, 212003, P.R. China

c School of life Science & Technology, China Pharmaceutical University, Nanjing 210009, P.R. China)

d Department of Biochemical Engineering, Nanjing university of Science & Technology, 200 Xiaolinwei, Nanjing, 210094, P.R. China xiaofengbao@mail.njust.edu.cn

Hg^{2+} and Ag^+ are two of the heavy transitional metals that receive intensive attention because large quantities of them are released into environment each year. Ag^+ is widely used in the electrical industry, photographic and imaging industry leading to environmental pollution^[1]. Exposure to mercury, even in small dose, is a great danger to humans and wildlife. When mercury enters the body it acts as a neurotoxin, harming the brain and nervous system^[2]. Mercury exposure is especially dangerous to pregnant women and young children^[3]. Thus development of sensitive and selective methods to determine trace Hg^{2+} and Ag^+ is of great interest. Conventional methods, such as atomic absorption spectroscopy^[4] and inductively coupled plasma mass spectroscopy (ICPMS)^[5], have been used in the past for this purpose. Although these methods are sensitive and accurate, advanced instrumentation and complicated time-consuming sample pre-treatments are needed. Fluorescence spectroscopy is a rapid nondestructive and relatively low cost method that can be used for real time tracking to detect ions in living systems^[6, 7].

Various fluorescent chemosensors for Hg^{2+} have been developed, such as those based on the receptor moiety of 3,9-dithia-6-azaundecane^[8], 2,6-bis (amino- methyl)pyridine^[9], oligonucleotide^[10], thioether-rich crown^[11], thiosemicarbazide^[12], 1,4-disubstituted azine^[13], thiol ligands on nanoparticle surface^[14], carbohydrazone^[15], cyclen^[16], thymine^[17] and etc. Chemosensors designed for Hg^{2+} usually contains thio groups because their high affinity for Hg^{2+} . A number of fluorescence chemosensors for Ag^+ containing receptor moiety of guanine^[18], piperazine^[19], benzoylthiourea^[20], DNA^[21], carbodithioate^[22] and 1,2-diphenyldiselenide^[23] have also been developed. Meanwhile there are several fluorescence chemosensors that can detect both Hg^{2+} and Ag^+ simultaneously, but most of them cannot distinguish one from another^[24-32].

In a previous study, FLTC was synthesized for the detection of HOCl ^[33]. In this study, we demonstrate that it can be used for the detection of Hg^{2+} and Ag^+ in buffers and living cells, which may find applications in fast detection of

Hg²⁺ and Ag⁺ or cell imaging.

Fig 1 chemical structure of FLTC

FLTC was synthesized according to a **previously** described procedure^[33], and its structure was clearly confirmed by ¹H NMR, ¹³C NMR and HRMS (Fig. S1-S3). At certain pH values, fluorescein compounds may undergo a ring open reaction, and emit strong fluorescence, **which causes** background interference **during** detection^[34, 35]. As shown in Fig. S4, an acid-base titration study conducted in **ethanol-H₂O** (3:2, v/v) solution showed **that** FLTC did not emit any distinct and characteristic fluorescence in the pH range of 1.0-7.0, which indicates **that** FLTC mainly presents **in its** spiro lactam form in acidic environments. When the pH was adjusted to values between 7.0 and 11.0, the fluorescence intensity at 524 nm was greatly enhanced, **likely** due to the ring-opening process of the spirocyclic moiety of fluorescein. **At a** pH was higher than 11.0, the fluorescence intensity tended to stabilize, which indicates **that** the open-ring form of FLTC became a dominant species in solution. These results showed that FLTC **is** insensitive to pH changes **from** 1.0 to 7.0 and may work under physiological conditions with very low background fluorescence. Therefore, an ethanol-H₂O (3:2, v/v, pH 7.15, HEPES buffer, 0.5mM) solution has been chosen for the following studies.

The fluorescence spectra of FLTC in the presence of metal ions (Ag^+ , Al^{3+} , Ba^{2+} , Ca^{2+} , Cd^{2+} , Co^{2+} , Cr^{3+} , Ir^{3+} , Cu^+ , Cu^{2+} , Fe^{2+} , Fe^{3+} , Hg^{2+} , K^+ , Li^+ , Mg^{2+} , Mn^{2+} , Na^+ , Ni^{2+} , Pb^{2+} , Sn^{2+} and Zn^{2+}) are shown in figure 2. A remarkable enhancement of fluorescence was observed only in the presence of Hg^{2+} (50 μM , 5eq.), suggesting that FLTC can be used to sense Hg^{2+} ions as a “turn on” chemosensor. The

mechanism may be explained by the formation of a strongly fluorescent ring-opened FLTC- Hg^{2+} complex (Fig 3) [36].

Fig. 2. The fluorescence spectra of FLTC (10 μM) in an ethanol- H_2O (3:2, v/v, pH 7.15, HEPES buffer, 0.5mM) solution in the absence and presence of 10 equivalents of metal ions.

Fig. 3 Proposed FLTC- Hg^{2+} complex

To gain a further insight into the complexation of FLTC with Hg^{2+} , fluorescence spectra of FLTC were recorded during titrating with Hg^{2+} in an ethanol- H_2O (3:2, v/v, pH 7.15, HEPES buffer, 0.5 mM) solution, and the excitation wavelength was 490nm. As shown in Fig. 4, with an increasing concentration of Hg^{2+} from 0 to 300 μM , the intensity at 524 nm was significantly increased, and reached a maximum at 30 equivalents of Hg^{2+} . To determine the complexation stoichiometry of the FLTC- Hg^{2+} , a Job's plot was generated by continuously varying the mole fraction of Hg^{2+} from 0 to 1 in a solution of $[\text{Hg}^{2+}] + [\text{FLTC}]$ with a total concentration of 50 μM . Analysis of the Job's plot revealed a maximum at an approximately 0.5 mole fraction, which

indicates a 1:1 stoichiometry for the FLTC-Hg²⁺ complex (Fig. S5). The binding association constant for the FLTC-Hg²⁺ complex was $2.2078 \times 10^4 \text{ M}^{-1}$, which was calculated from the titration data using a Benesi-Hildebrand plot ^[31, 33] (Table S1, Fig.S6). The detection limit ^[31, 33] of FLTC was found to be 0.21 μM (Table S2, Fig. S7). The previous reported detection limits for Hg²⁺ showed in Tab.1. A competition study was also carried out by pre-addition of different metal ions. As shown in Fig. S8, in the presence of Ag⁺, the addition of Hg²⁺ led to a great enhancement of fluorescence intensity while the presence of other metal ions completely (Pb²⁺, Fe³⁺, Sn²⁺) or significantly (Cr³⁺, Cu²⁺, Fe²⁺, Mn²⁺) quenched the fluorescence.

Fig 4. Fluorescence spectra of FLTC (10 μM) in an ethanol-H₂O (3:2, v/v, pH 7.15, HEPES buffer, 0.5 mM) solution when different amounts of Hg²⁺ were added.

The sensing ability of the FLTC-Hg²⁺ complex towards metal ions (Ag⁺, Al³⁺, Ba²⁺, Ca²⁺, Cd²⁺, Co²⁺, Cr³⁺, Cu⁺, Cu²⁺, Fe²⁺, Fe³⁺, Hg²⁺, K⁺, Li⁺, Mg²⁺, Mn²⁺, Na⁺, Ni²⁺, Ir³⁺, Sn²⁺ and Zn²⁺) was shown in fig. 5, indicates a significant increase of fluorescence intensity only for Ag⁺. These results demonstrate that the FLTC-Hg²⁺ complex can function as a highly selective fluorescent chemosensor for Ag⁺ detection over various other metal ions.

(a)

(b)

Fig. 5 Fluorescence spectra of FLTC (10 μM) with Hg^{2+} (50 μM , 5 equiv) in ethanol- H_2O (3:2, v/v, pH 7.15, HEPES buffer, 0.5 mM) in the presence of 5 equiv of metal ions (a). Bar graph shows the relative emission intensity of $\text{FLTC}\cdot\text{Hg}^{2+}$ complex at 524 nm upon the addition of metal ions (b).

Fluorescence spectra of the $\text{FLTC}\cdot\text{Hg}^{2+}$ complex were also monitored during titrating with Ag^+ in an ethanol- H_2O (3:2, v/v, pH 7.15, HEPES buffer, 0.5 mM) solution. As shown in Fig. S9, with the addition of Ag^+ ions, a gradual increase in fluorescence was found, which reached a maximum at an Ag^+ ion concentration of 100 μM . The detection limit of $\text{FLTC}\cdot\text{Hg}^{2+}$ for Ag^+ was also calculated from the

titration, the calculated detection limit was 0.009 μM (Table S3, Fig. S10), which was lower than most reported detection limits (Tab. 2). A competition study was also carried out by pre-addition of different metal ions. As shown in Fig. S11, most pre-added ions do not cause an appreciable change of emission intensity except for Cr^{3+} , Fe^{3+} and Sn^{2+} (Fig. S11). The possible Ag^+ sensing mechanism of $\text{FLTC} \cdot \text{Hg}^{2+}$ seems to be in agreement with the general knowledge of the unstable Schiff base^[37], when Hg^{2+} and Ag^+ are both present in a solution C=N tends to be broken. Thus, amines will be released, which was confirmed by MS (Fig. S12), and the amine may undergo a ring opening process to emit strong fluorescence.

Tab.1 Different detection Limit of Hg^{2+}

Tab.2 Different detection Limit of Ag^+

COMPOUND	Detection Limit of	COMPOUND	Detection Limit of Ag^+
FTLC	0.21 μM	FTLC	9nM
1	0.010 μM ^[9]	1	200nM ^[19]
MF1	0.060 μM ^[11]	SC1	52nM ^[23]
2	4.60 μM ^[13]	HTMIX	80 nM ^[36]
Complex 1	0.118 μM ^[30]	Complex 1	112nM ^[30]
SQ1	0.13 μM ^[31]	TPE-4DDC	874nM ^[38]

We further evaluated the applicability of FLTC as a fluorescent probe for Hg^{2+} and Ag^+ detection through *in vitro* cell studies. To evaluate the cytotoxicity of FLTC, the cell viability was determined using a MTT assay in L-02 cells with FLTC concentrations of 0-80 μM (Fig. S13). When FLTC was less than 5 μM , almost no cell toxicity was detected. Therefore, the following cell studies were carried out using 5 μM FLTC. The cells were incubated with 25 μM HgCl_2 0.5mM HEPES buffer for 30min at room temperature and subsequently incubated with the probe 5 μM FLTC at 37°C under 5% CO_2 for additional 2h. The cells were further incubated with 25 μM AgNO_3 0.5mM HEPES buffer for 30min at room temperature followed by incubated at 37°C under 5% CO_2 for another 2h. No fluorescence was observed when cells were treated

only with DMSO or FLTC (Fig. 6 b, d). However, when an aqueous solution of HgCl_2 was added before loading with FLTC, cell emitted green fluorescence ($\lambda_{\text{ex}} = 490 \text{ nm}$) at 524 nm (Fig. 6f), which was greatly enhanced after a subsequent addition of AgNO_3 (Fig. 6h). Therefore, FLTC can be used as a fluorescent probe to detect both Hg^{2+} and Ag^+ in human L-02 liver cells.

Fig. 6 Fluorescence images of human L-02 hepatocytes incubated with FLTC and Hg^{2+} and/or Ag^+ . The DMSO-treated cells were taken as a control (a: bright-field image; b: fluorescence image), and only FLTC-treated cells were also taken as a control (c: bright-field image; d: fluorescence image). The L-02 cells were incubated with 25 μM HgCl_2 in 0.5mM HEPES buffer for 30 min at room temperature, followed by 5 μM FLTC for additional 2h (e: bright-field image; f: fluorescence image), further incubated with 25 μM AgNO_3 for 2h (g: bright-field image; h: fluorescence image).

In summary, a fluorescein derivative FLTC was synthesized as a selective chemosensor for Hg^{2+} and Ag^+ in an ethanol- H_2O (3:2, v/v, HEPES buffer, 0.5mM, pH 7.15) solution. FLTC and the FLTC- Hg^{2+} complex could detect Hg^{2+} and Ag^+ respectively. The FLTC- Hg^{2+} complex has a low Ag^+ detection limit of 0.009 μM . Fluorescence microscopy experiments further demonstrated that FLTC can be used as a fluorescent probe to detect Hg^{2+} and Ag^+ in human liver cells (L-02).

Acknowledgements

This research was supported by National Science Foundation of China (21302098), the Fundamental Research Funds for the Central Universities (KJQN201415).

References

1. Pedroso M S, Pinho G L L, Rodrigues S C, et al. Mechanism of acute silver toxicity in the euryhaline copepod *Acartia tonsa*. *Aquatic toxicology*, 2007, 82(3): 173-180.8.
2. Tchounwou P B, Ayensu W K, Ninashvili N, et al. Review: Environmental exposure to mercury and its toxicopathologic implications for public health. *Environmental Toxicology*, 2003, 18(3): 149-175.
3. Renzoni A, Zino F, Franchi E. Mercury levels along the food chain and risk for exposed populations. *Environmental Research*, 1998, 77(2): 68-72.
4. Gómez-Ariza J L, Lorenzo F, García-Barrera T. Comparative study of atomic fluorescence spectroscopy and inductively coupled plasma mass spectrometry for mercury and arsenic multispeciation. *Analytical and bioanalytical chemistry*, 2005, 382(2): 485-492.
5. Acon B W, McLean J A, Montaser A. A large bore-direct injection high efficiency nebulizer for inductively coupled plasma spectrometry. *Analytical chemistry*, 2000, 72(8): 1885-1893.
6. Kim H N, Lee M H, Kim H J, et al. A new trend in rhodamine-based chemosensors: application of spirolactam ring-opening to sensing ions. *Chemical Society Reviews*, 2008, 37(8): 1465-1472.
7. Domaille D W, Que E L, Chang C J. Synthetic fluorescent sensors for studying the cell biology of metals. *Nature Chemical Biology*, 2008, 4(3): 168-175.
8. Nolan E M, Lippard S J. A "turn-on" fluorescent sensor for the selective detection of mercuric ion in aqueous media. *Journal of the American Chemical Society*, 2003, 125(47): 14270-14271.
9. Guo X, Qian X, Jia L. A highly selective and sensitive fluorescent chemosensor for Hg²⁺ in neutral buffer aqueous solution. *Journal of the American Chemical Society*, 2004, 126(8): 2272-2273.
10. Ono A, Togashi H. Highly selective oligonucleotide-based sensor for mercury (II) in aqueous solutions. *Angewandte Chemie International Edition*, 2004, 43(33): 4300-4302.
11. Yoon S, Albers A E, Wong A P, et al. Screening mercury levels in fish with a selective fluorescent chemosensor. *Journal of the American Chemical Society*, 2005, 127(46): 16030-16031.
12. Yang Y K, Yook K J, Tae J. A rhodamine-based fluorescent and colorimetric chemodosimeter for the rapid detection of Hg²⁺ ions in aqueous media. *Journal of the American Chemical Society*, 2005, 127(48): 16760-16761.

13. Caballero A, Martínez R, Lloveras V, et al. Highly selective chromogenic and redox or fluorescent sensors of Hg^{2+} in aqueous environment based on 1, 4-disubstituted azines. *Journal of the American Chemical Society*, 2005, 127(45): 15666-15667.
14. Huang C C, Chang H T. Selective gold-nanoparticle-based "turn-on" fluorescent sensors for detection of mercury (II) in aqueous solution. *Analytical chemistry*, 2006, 78(24): 8332-8338.
15. Wu D, Huang W, Duan C, et al. Highly sensitive fluorescent probe for selective detection of Hg^{2+} in DMF aqueous media. *Inorganic chemistry*, 2007, 46(5): 1538-1540.
16. Shiraishi Y, Sumiya S, Kohno Y, et al. A rhodamine- cyclen conjugate as a highly sensitive and selective fluorescent chemosensor for Hg (II). *The Journal of organic chemistry*, 2008, 73(21): 8571-8574.
17. Tang Y, He F, Yu M, et al. A reversible and highly selective fluorescent sensor for mercury (II) using poly (thiophene) s that contain thymine moieties. *Macromolecular rapid communications*, 2006, 27(6): 389-392.
18. Chen X, Chen Y, Zhou X, et al. Detection of Ag^+ ions and cysteine based on chelation actions between Ag^+ ions and guanine bases. *Talanta*, 2013, 107: 277-283.
19. Li C Y, Kong X F, Li Y F, et al. Ratiometric and colorimetric fluorescent chemosensor for Ag^+ based on tricarbo-cyanine. *Dyes and Pigments*, 2013, 99(3): 903-907
20. Yang Z, She M, Yin B, et al. Solvent-dependent turn-on probe for dual monitoring of Ag^+ and Zn^{2+} in living biological samples. *Analytica chimica acta*, 2015, 868: 53-59.
21. Zhao C, Qu K, Song Y, et al. A Reusable DNA Single - Walled Carbon - Nanotube - Based Fluorescent Sensor for Highly Sensitive and Selective Detection of Ag^+ and Cysteine in Aqueous Solutions. *Chemistry-A European Journal*, 2010, 16(27): 8147-8154.
22. Wang M X, Meng X M, Zhu M Z, et al. Novel fluorescent chemosensor for Ag^+ based on coumarin fluorophore. *Chinese Chemical Letters*, 2008, 19(8): 977-980
23. Huang S, He S, Lu Y, et al. Highly sensitive and selective fluorescent chemosensor for Ag^+ based on a coumarin-Se2N chelating conjugate. *Chemical Communications*, 2011, 47(8): 2408-2410
24. Yang H, Zhou Z, Li F, et al. New Hg^{2+} and Ag^+ selective colorimetric sensor based on thiourea subunits. *Inorganic Chemistry Communications*, 2007, 10(10): 1136-1139
25. Liu L, Zhang G, Xiang J, et al. Fluorescence "turn on" chemosensors for Ag^+ and Hg^{2+} based on tetraphenylethylene motif featuring adenine and thymine moieties. *Organic letters*, 2008, 10(20): 4581-4584.
26. Freeman R, Finder T, Willner I. Multiplexed analysis of Hg^{2+} and Ag^+ ions by nucleic acid functionalized CdSe/ZnS quantum dots and their use for logic gate operations. *Angewandte Chemie International Edition*, 2009, 48(42): 7818-7821.
27. Hung H C, Cheng C W, Wang Y Y, et al. Highly Selective Fluorescent Sensors for Hg^{2+} and Ag^+ Based on Bis-triazole-Coupled Polyoxyethylenes in MeOH Solution. *European Journal of Organic Chemistry*, 2009, 2009(36): 6360-6366.
28. Lin Z, Li X, Kraatz H B. Impedimetric immobilized DNA-based sensor for simultaneous detection of Pb^{2+} , Ag^+ , and Hg^{2+} . *Analytical chemistry*, 2011, 83(17): 6896-6901.
29. Hao C, Xua L, Xing C, et al. Oligonucleotide-based fluorogenic sensor for simultaneous detection of heavy metal ions. *Biosensors and Bioelectronics*, 2012, 36(1): 174-178.
30. Khatua S, Schmitt M. A single molecular light-up sensor for quantification of Hg^{2+} and Ag^+

- in aqueous medium: high selectivity toward Hg^{2+} over Ag^+ in a mixture. *Organic letters*, 2013, 15(17): 4422-4425.
31. Fan J, Chen C, Lin Q, et al. A fluorescent probe for the dual-channel detection of $\text{Hg}^{2+}/\text{Ag}^+$ and its Hg^{2+} -based complex for detection of mercapto biomolecules with a tunable measuring range. *Sensors and Actuators B: Chemical*, 2012, 173: 874-881.
 32. Lou T, Chen Z, Wang Y, et al. Blue-to-red colorimetric sensing strategy for Hg^{2+} and Ag^+ via redox-regulated surface chemistry of gold nanoparticles. *ACS applied materials & interfaces*, 2011, 3(5): 1568-1573.
 33. Jin X, Hao L, Hu Y, et al. Two novel fluorescein-based fluorescent probes for hypochlorite and its real applications in tap water and biological imaging. *Sensors and Actuators B: Chemical*, 2013, 186: 56-60.
 34. Pullela P K, Chiku T, Carvan M J, et al. Fluorescence-based detection of thiols in vitro and in vivo using dithiol probes. *Analytical biochemistry*, 2006, 352(2): 265-273.
 35. Wang D, Xiang X, Yang X, et al. Fluorescein-based chromo-fluorescent probe for zinc in aqueous solution: Spirolactam ring opened or closed? *Sensors and Actuators B: Chemical*, 2014, 201: 246-254.
 36. Lin D S, Lai J P, Sun H, et al. A turn-on fluorescein spirolactam derivative as a high selective fluorescence probe for detection of silver ion (I) in water. *Analytical Methods*, 2014, 6(5): 1517-1522.
 37. Solomons, T. W. G. *Organic chemical*. New York: Wiley 1980. (pp. 703-714).
 38. Li Y, Yu H J, Shao G, Gan F. A tetraphenylethylene-based "turn on" fluorescent sensor for the rapid detection of Ag^+ ions with high selectivity. *Journal of Photochemistry and Photobiology A: Chemistry* 301 (2015) 14-19

FLTC was synthesized and used as a fluorescent chemosensor to detect Hg^{2+} . It showed high selectivity toward Hg^{2+} over many heavy metal ions in an ethanol- H_2O (3:2, v/v, HEPES buffer, 0.5mM, pH 7.15) solution with a detection limit of 0.21 μM . After complexation with Hg^{2+} , FLTC showed extremely high selectivity toward Ag^+ with a detection limit of 0.009 μM . Therefore, detection of Hg^{2+} and Ag^+ could be realized using FLTC and the FLTC- Hg^{2+} complex, respectively. Cytotoxicity assays and fluorescence microscopy analysis showed that FLTC could be used as a fluorescent probe to detect Hg^{2+} and Ag^+ in L-02 human liver cells.