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COMMUNICATION

Fluorescent chemodosimeter for Cys/Hcy with a large absorption shift and imaging in living cells†

Mingming Hu,[a] Jiangli Fan,*[a] Honglin Li,[a] Kedong Song,[b] Song Wang,[a] Guanghui Cheng[a] and Xiaojun Peng*[a]

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A novel molecule T1 with efficient intramolecular charge transfer was designed as a fluorescent chemodosimeter for cysteine (Cys) and homocysteine (Hcy). Upon addition of Cys/Hcy, T1 exhibited greatly enhanced fluorescence intensity as well as a large absorption peak shift (70 nm), and can be used for bioimaging of Cys/Hcy in living cells and detection in human plasma by visual color change. The detection mechanism was proved by ^{1}H NMR, mass spectrometry analysis and Gaussian calculations.

For participation in the process of reversible redox reaction, mercapto biomolecules play a crucial role in maintaining biological redox homeostasis,[1] in which cysteine (Cys) and homocysteine (Hcy) are essential biological molecules relevant to the growth of cells and tissues in living systems.[2] Cys deficiency is involved in many syndromes, for instances, slow growth in children, liver damage, skin lesions and weakness.[3] Hcy has also been linked to increased risk of Alzheimer's disease,[4] inflammatory bowel disease and osteoporosis.[5]

Recently, a lot of effort has gone into the development of selective fluorescent bioimaging for highly sensitive, high-speed spatial analysis of cells. Chemodosimeters, which are based on an irreversible or essentially irreversible reaction, have attracted much interest from researchers. Compared with chemosensors, chemodosimeters have advantages in terms of selectivity and sensitivity, and their accumulative effect plays an important role in detection of analytes.[6] To image the distribution of Cys/Hcy in cellular processes, some suitable turn-on fluorescent chemodosimeters for Cys/Hcy have been developed.[7] For example, works based on either the nucleophilic addition of thiol,[8–25] cyclization with aldehydes,[26–41] or cleavage by thiols,[42–51] are all significant for the detection of mercapto biomolecules.

Ratiometric probes allow measurement at two different wavelengths, which could provide a built-in correction for environmental effects and improve the detection sensitivity.[52–55] Thus, a chemodosimeter exhibiting greatly changed emission intensity as well as a large absorption peak shift is ideal for sensing. After Rusin and Strongin *et al.* designed the first fluorescent chemodosimeter for Cys/Hcy by colorimetric and fluorometric responses with excitation in the visible region,[26] some relative works have been reported.[29,30,33,36] However, they have not achieved intracellular detection of Cys/Hcy yet. It still leaves significant work to explore novel ratiometric chemodosimeters in the visible region with appropriate membrane permeability.

In our previous study of a novel type of squarylium infrared absorbing dye,[56] we found that, part of the dye, 7-dimethylamino-1,4-benzoxazin-2-one showed good spectral properties. This small molecule is not symmetrical, and the dipole moment in the excited state is much larger than in the ground state.[57] The slight structure differences between coumarin and 7-dimethylamino-1,4-benzoxazin-2-one, make the latter have a longer wavelength. Therefore, we introduced a simple aldehyde group to this molecule and developed a novel fluorescent chemodosimeter **T1** which displayed absorption ratiometric and fluorescence enhanced responses to Cys/Hcy based on cyclization of mercapto biomolecules with aldehydes.[39] (Fig. 1) In this molecule, the first singlet state offers an internal charge transfer, owing to conjugation between the electron-donor amino substituent in the 7-position (Scheme S1†), and the electron withdrawing carbonyl group of the heterocycle. Consequently, a large Stokes shift between absorbance and fluorescence maxima is expected.[58] To the best of our knowledge, it is the first fluorescent chemodosimeter based on the 7-dimethylamino-1,4-benzoxazin-2-one dyes.

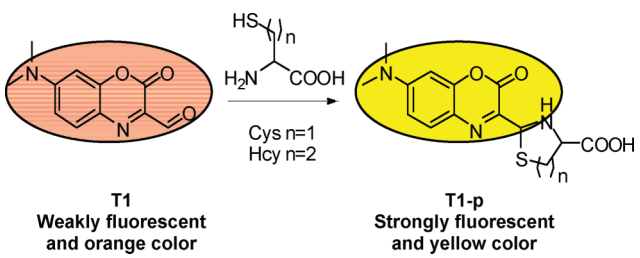

Fig. 1 Reaction of aldehyde with N-terminal Cys residue to form thiazolidine.

Herein, the recognition of Cys/Hcy with **T1** was investigated in a mixture of acetonitrile and HEPES (3 : 7, v/v) solution at pH 7.4 by UV–vis absorption and fluorescence techniques.

[a]*State Key Laboratory of Fine Chemicals, Dalian University of Technology, Dalian 116012, China. E-mail: pengxj@dlut.edu.cn, fanjl@dlut.edu.cn; Fax: +86-411-3989-3906; Tel: +86-411-3989-3899*

[b]*Dalian R&D Center for Stem Cell and Tissue Engineering, Dalian University of Technology, Dalian, 116023, China*

† Electronic supplementary information (ESI) available: Synthetic procedure, **^{1}H NMR**, **^{13}C NMR** and data of **T1**. **^{1}H NMR** and **TOF-MS** of products of reaction of **T1** with Cys. Fluorescence spectra and calculation in the assay. Cell incubation and fluorescence imaging. See DOI: 10.1039/c0ob00957a

The pH independent spectra show that **T1** can work in a wide physiological pH range (Figure S1†). All the tests were taken 1 h later after addition of the Cys/Hcy to ensure the reaction reaching equilibrium (Figure S2, S3†).

The free **T1** is weakly fluorescent in pH 3–8, a wide physiological pH range. Upon addition of Cys, the fluorescence increased rapidly, peaking at λ_{em} = 560 nm. (Fig. 2a) After 50 equiv Cys were added to the solution of **T1**, about 5-fold enhancement in fluorescence was observed at room temperature in acetonitrile–HEPES buffer (20 mM, pH = 7.4) solution (3 : 7, v/v). The fluorescence intensities of **T1** at 560 nm show a good linear relationship with the concentration of Cys, using physiological levels ranging from 0 to 500 μM[58,59] (the total concentration of Cys in healthy plasma is in the range of 240 μM–360 μM) (Fig. 2b). The intensity is also linear in response to lower concentrations of Cys/Hcy (Figure S4, S5†). Its detection limit for Cys is 6.8×10^{-7} M in acetonitrile–HEPES.

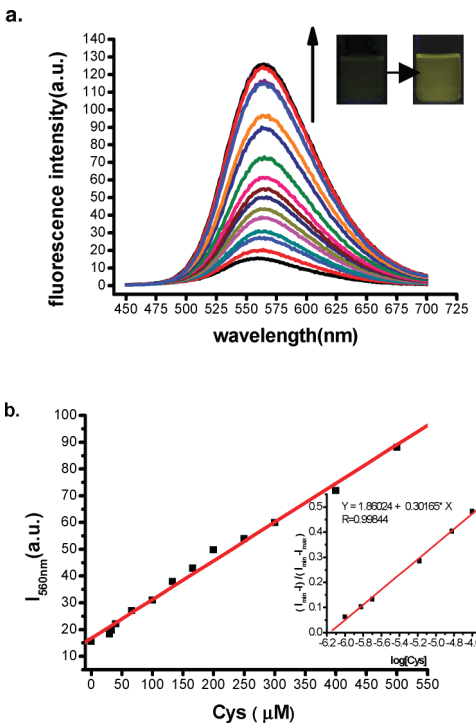

Fig. 2 (a). Fluorescence spectral changes of **T1** (10 μM) upon addition of Cys (0–3000 μM). Each spectrum was recorded after 60 min. Inset shows the fluorescence changes of **T1** (10 μM) in the absence of Cys (left) and presence of 500 μM Cys (right). (b) Fluorescence intensity at 560 nm of **T1** (10 μM) upon addition of Cys (0–500 μM). Inset shows the detection limit for Cys. Conditions: acetonitrile–HEPES buffer (20 mM, pH = 7.4) solution (3 : 7, v/v, rt), λ_{ex} = 430 nm.

As displayed in Fig. 3a, upon addition of Cys, the absorption band of **T1** centered at 500 nm gradually decreased and a new absorption band centered at 430 nm appeared with a distinct isosbestic point at 452 nm, corresponding to an apparent color change from orange to yellow (Figure S7†). This fact indicates that **T1** can serve as a "naked-eye" chemodosimeter for Cys/Hcy.

A typical calibration graph of the absorption response of **T1** to Cys under the optimum experimental conditions was obtained as shown in Fig. 3b. The absorbance ratios at 500 nm and 430 nm are linearly related to the Cys concentration between 0–500 μM,

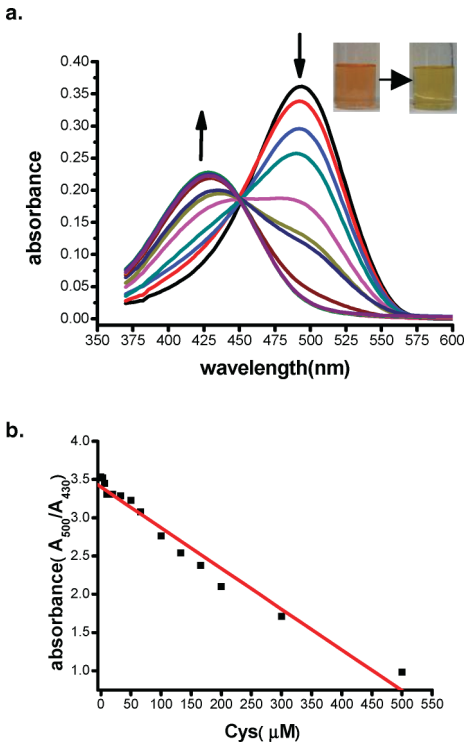

Fig. 3 (a). Absorption spectral changes of **T1** (10 μM) upon addition of Cys (0–2500 μM). Each spectrum was recorded after 60 min. Inset shows the color changes of **T1** (10 μM) in the absence of Cys (left) and presence of 500 μM Cys (right). (b) Plot of the absorption intensity ratios at 500 nm and 430 nm of **T1** (10 μM) upon addition of Cys (0–500 μM). Conditions: acetonitrile–HEPES buffer (20 mM, pH = 7.4) solution (3 : 7, v/v, rt).

which means it can detect the normal human physiological Cys concentration.[4,59] An obvious color change was also similarly seen upon addition of Hcy (Figure S7†)

The fluorescence responses of **T1** (10 μM) to various amino acids and thiol biomacromolecules (50 equiv.) were also investigated, such as glycine: Gly, alanine: Ala, valine: Val, leucine: Leu, isoleucine: Ile, and proline: Pro, polar groups (serine: Ser, threonine: Thr) including glutathione (GSH), and acidic (glutamic acid: Glu) and basic (arginine: Arg, lysine: Lys) side chains. None of the amino acids except Hcy and Cys induced any fluorescence intensity enhancement. (Fig. 4) Importantly, as a structurally related thiol biomolecule, reduced glutathione (GSH) hardly reacted with **T1** under these conditions and did not exhibit any absorption and fluorescence signal changes, which may be due to its steric hindrance.

To confirm the formation of thiazolidine after addition of Cys/Hcy to **T1**, the partial ^{1}H NMR spectra of the reaction "**T1-p**" of **T1** with Cys are shown in Fig. 5. The resonance signal corresponding to the aldehyde proton at 9.83 ppm disappeared; however, concomitantly, two new peaks at 5.81 and 5.64 ppm assigned to the methine proton of the thiazolidine diastereomer emerged. This formation was further characterized by mass spectrometry analysis (Figure S12†). Q-TOF MS:$[T1\text{-}p\text{-}H]^-$ 320.0705; found 320.0713.

Meanwhile, the calculation based on density functional theory (DFT) for **T1** and the product **T1-p** was performed to further understand the dependence of the CHO group on photophysical

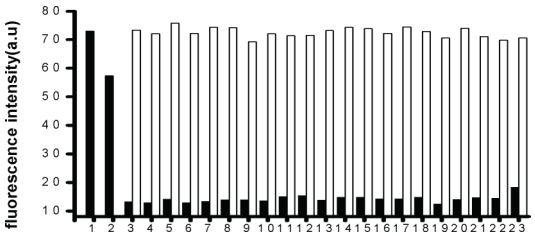

Fig. 4 Fluorescence responses of **T1** (10 μM) to various bioanalytes at 560 nm. Each spectrum was recorded after 60 min. Conditions: acetonitrile–HEPES buffer (20 mM, pH = 7.4) solution (3 : 7, v/v, rt), λ_{ex} = 430 nm. Black bars represent the addition of various bioanalytes to **T1** solution. White bars represent the addition of Cys (500 μM) to the above solutions, respectively. λ_{ex} = 430 nm. 1. Cys, 2. Hcy, 3. Ala, 4. Ser, 5. Trp, 6. Met, 7. Gln, 8. Val, 9. Arg, 10. Phe, 11. Pro, 12. Thr, 13. Gly, 14. Lys, 15. His, 16. Asp, 17. Ile, 18. Asn, 19. Leu, 20. Glu, 21. cystine, 22. Tyr, 23. GSH.

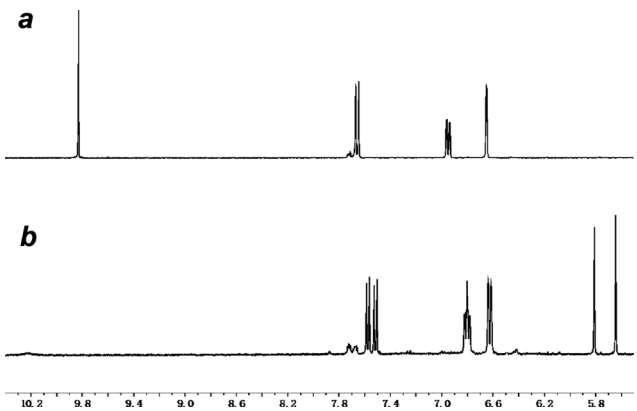

Fig. 5 ^{1}H NMR (400 MHz) spectra of sensor **T1** in DMSO-d_6 (a) and **T1-p** in DMSO-d_6 (b).

properties. Comparing the level changes of the HOMO (the highest occupied molecular orbitals) and LUMO (the lowest unoccupied molecular orbitals) in **T1** and **T1-p**, respectively, (Fig. 6) due to the ICT effect, after reaction with Cys/Hcy both of them increased. As the LUMOs increased much more, the HOMO–LUMO energy gaps were calculated as 3.35 eV and 3.39 eV for **T1** and **T1-p**, respectively, which is in agreement with the remarkable blue-shift in the absorption spectra. These results from the spectra and the Gaussian calculation suggest that the rational design of this fluorophore can be used for designing new fluorescent probes.

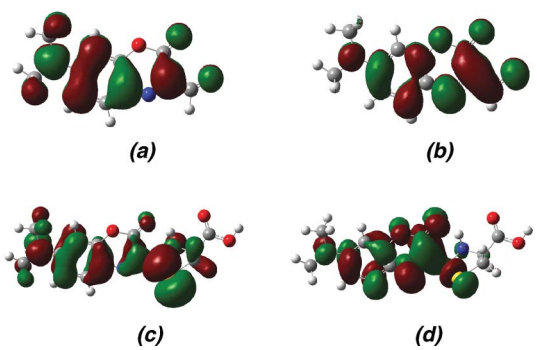

Fig. 6 The HOMOs of **T1** (a), and LUMOs of **T1** (b), HOMOs of **T1-p** (c), LUMOs of **T1-p** (d).

The detection of GSH in human plasma without prior deproteinization is an important practical application of **T1** for Cys/Hcy (Figure S8†). In our tests, commercial native human blood plasma samples were simply reconstituted with HEPES buffer (pH 7.4). The samples were spiked with Hcy, Cys, and GSH respectively. In the presence of **T1**, upon adding trifluoroacetic acid to precipitate protein, the samples afforded selective yellow color formation in the presence of Cys and Hcy, while the plasma with GSH still showed the orange color of **T1**. This result is completely consistent with our prior results in buffer solutions. The results in native plasma solutions show its potential to be used in a physiological environment.[12,26,60]

To test the capability of **T1** to image Cys in living cells, we further applied **T1** to osteoblast cells. After incubation with 10 μM **T1** in culture medium at 37 °C for 20 min, osteoblast cells showed marked intracellular fluorescence (Fig. 7a, 7b). In contrast, when cells were not incubated with **T1**, there was no intracellular background fluorescence under the same bioimaging conditions (Fig. 7c, 7d). The results suggest that **T1** can easily penetrate cell membranes and make fast fluorescent labeling. Considering the relationship between Cys levels and many diseases, this probe may offer a simple and visible way to detect Cys *in vivo* at real time.

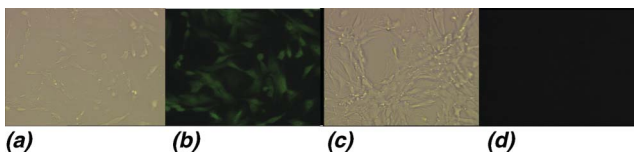

Fig. 7 Fluorescence images of osteoblasts cells incubated with (a, b) or without (c, d) 10 μM **T1** at 37 °C. After 20 min incubation: (a) brightfield image of cells; and (b) excited in 365 nm; (c) scanning was taken on brightfield; (d) excited at 365 nm, no obvious fluorescence was observed.

In summary, we have developed a specific chemodosimeter **T1** which can be used for selective bioimaging of Cys/Hcy over other amino acids in living cells and detection in human plasma by visual color change. To the best of our knowledge, it is the first fluorescent chemodosimeter based on the 7-dimethylamino-1,4-benzoxazin-2-one dyes.

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