Electronic Supplementary Information

A facile approach for sensitive, reversible and ratiometric detection of biothiols based on thymine-mediated excimer/monomer transformation

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Experimental Section

Materials and reagents: 1-pyrenemethanol, phosphorus tribromide, thymine, potassium carbonate (K_2CO_3), 4(-2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), glutathione (GSH), cysteine (Cys), homocysteine (Hcy), and various amino acids: L-serine (Ser), L-tryptophan (Trp), L-phenylalanine (Phe), L-tyrosine (Tyr), L-glutamic acid (Glu), L-arginine (Arg), L-histidine (His), L-lysine (Lys), L-aspartic acid (Asp), L-threonine (Thr), L-asparagine (Asn), L-glutamine (Gln), glycine (Gly), L-proline (Pro), L-alanine (Ala), L-isoleucine (IIe), L-leucine (Leu), L-methionine (Met) and L-valine (Val) were purchased from Sigma-Aldrich Co. Mercury(II) perchlorate trihydrate ($Hg(ClO_4)_2{\cdot}3H_2O$) was obtained from Alfa. Human urine was from a healthy female. The buffer solutions include: acetic acid-sodium acetate buffer pH: 2.6, 3.8, 4.8 and 5.8; borax-boric acid buffer pH: 8.0, 8.4; 0.025 M HEPES buffer pH: 7.0; sodium phosphate dibasic-sodium phosphate monobasic buffer pH: 7.4. Tetrahydrofuran (THF), N, N-dimethylformamide (DMF) dimethyl sulfoxide (DMSO) was analytically pure solvents and distilled before use. Petroleum ether, diethyl ether, ethyl acetate were analytically pure solvents.

Synthesis of Compound PyT: 1-Bromomethylpyrene was obtained by a variation of the reported procedure (Reference: *J. Org. Chem.*, 2008, **73**, 8212–8218). Under nitrogen, 1-pyrenemethanol (0.2 g, 0.86 mmol) was dissolved in THF (1 mL), the mixture was cooled to 0 °C, followed by addition of phosphorus tribromide (100 μL, 1.05 mmol) via syringe. The mixture was stirred at room temperature for 2h, then the resulting mixture was filtered and the residue was washed with diethyl ether to yield

1-bromomethylpyrene as a yellow solid.

Compound PyT was synthesized as follows: Under nitrogen atmosphere, thymine (200 mg, 1.59 mmol) and K_2CO_3 (207 mg, 1.61 mmol) was dissolved in DMF (20 mL), then the above obtained 1-bromomethylpyrene in DMF (10 mL) was added, the reaction mixture was stirred at 90 °C for 24 h. After cooling to room temperature, the sediment was filtered, the solvent was then removed. The resulting solid was purified by column chromatography (petroleum ether: ethyl acetate = 2:3, v/v) to give PyT (135mg, 46.2 %). ^{1}H-NMR (400 MHz, DMSO-d_6, δ ppm): 11.46 (s, 1H), 8.46-8.43 (d, 1H), 8.36-8.30 (m, 4H), 8.22-8.17 (m, 2H), 8.13-8.09 (t, 1H),7.92-7.90 (d, 1H), 7.61 (s, 1H), 5.64 (s, 2H), 1.73 ppm (s, 3H). MS (ESI): m/z 342.9 [M^+].

Synthesis of complex PyT-Hg(II)-TPy: Under nitrogen, the above-obtained PyT (40 mg, 0.12mmol) was dissolved in dry THF (10 mL), afterwards $Hg(ClO_4)_2{\cdot}3H_2O$ (54 mg, 0.12 mmol) was added. The reaction mixture was stirred at room temperature for 3 h. Then the solvent was removed, the resulting solid was washed with distilled water for 5 times and dried under vacuum. ^{1}H-NMR (400 MHz, DMSO-d_6, δ ppm): 8.52-8.50 (d, 2H), 8.36-8.30 (m, 8H), 8.22-8.17 (m, 4H), 8.13-8.09 (t, 2H),7.96-7.94 (d, 2H), 7.64 (s, 2H), 5.70-5.65 (d, 4H), 1.79 ppm (s, 6H). MS (ESI): m/z 878.0 [M^+], 901.5 [$M+Na^+$].

Measurements: ^{1}H-NMR spectrum was recorded on a Bruker Avance 400MHz NMR Spectrometer. UV-vis spectra were recorded on a Hitachi U-3010 UV-vis Spectrophotometer. Fluorescence spectra were recorded on a Hitachi F-4600

fluorescence spectrophotometer with excitation wavelength being 345 nm. Mass spectra were obtained through Bruker Esquire HCT Plus mass spectrometer.

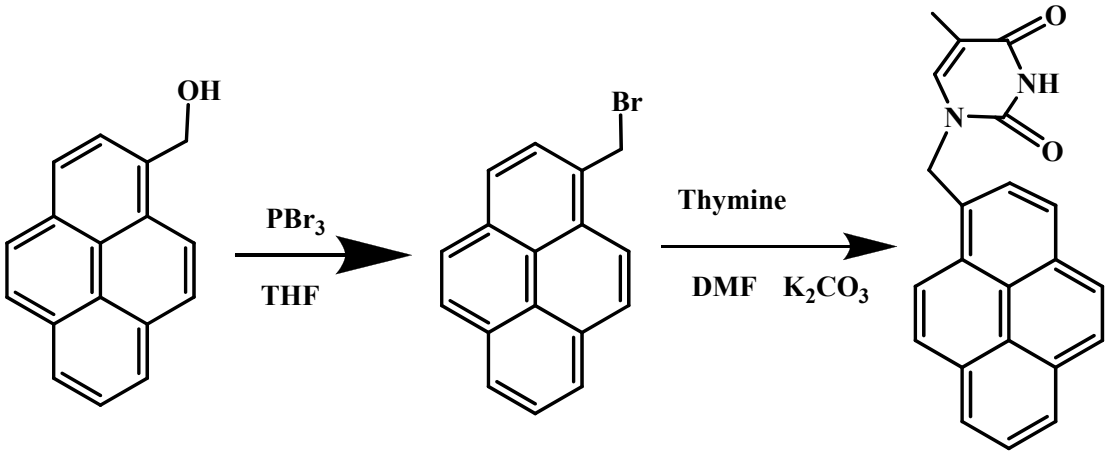
For thiol sensing, the stock solution of PyT (1 mM), PyT-Hg(II)-TPy (5×10^{-4} M) was prepared by dissolving PyT, PyT-Hg(II)-TPy in DMSO. Stock solutions of Hg^{2+}, GSH, Cys, Hcy and various other amino acids (1 mM) were prepared by dissolving them in water. The test solution was prepared by adding the requisite amounts of stock solutions together, then diluting with pH 7.0 HEPES buffer and DMSO, the final solvent was HEPES-buffered (pH 7.0) water-DMSO (7/1, v/v). Upon addition of analyte, the solution was stirred for 30 seconds, then the fluorescence spectra were recorded; unless specified otherwise, ex slit = 5 nm, em slit = 5 nm.

For the thiol sensing in urine sample, the urine sample collected from a healthy adult volunteer was used as the urine stock solution. The test solution was prepared by adding the requisite amounts of stock solutions together, then diluting with pH 7.0 HEPES buffer and DMSO, the final solvent was HEPES-buffered (pH 7.0) water-DMSO (7/1, v/v). The solution was stirred for 30 seconds, then the fluorescence spectra were recorded. The final urine concentration in the test solution is 50-fold diluted, since there are some thiols (up to 30 μM) in urine sample (B. Seiwert and U. Karst, *Anal. Chem.*, 2007, **79**, 7131; T. Ubuka, K. Kobayashi, K. Yao, H. Kodama, K. Fujii, K. Hirayama, T. Kuwari, S. Mizuhara, *Biochim. Biophys. Acta*, 1968, **158**, 493.).

For the thiol sensing in serum sample, fetal bovine serum was used. In serum, most of the thiol-containing amino acids are bound to proteins or other thiols and are

in the disulfide form (Nekrassova, O., Lawrence, N.S., Compton, R.G., 2003. *Talanta* 60, 1085-1095). After reduction with suitable reagents, these low-molecule mass thiols are bound free in the serum, which then can be used for analysis. In this study, the disulfide bonds were reduced in order to release the protein-bound thiols by addition of triphenylphosphine as catalyst according to literature report (Li Shang, Jianyuan Yin, Jing Li, Lihua Jin, Shaojun Dong, *Biosensors and Bioelectronics*, 2009, **25**, 269-274; J. V. Ros-Lis, B. Garcia, D. Jimenez, R. Martinez-Manez, F. Sancenon, J. Soto, F. Gonzalvo, M. Carmen Valldecabres, *J. Am. Chem. Soc.* 2004, **126**, 4064-4065). The final serum concentration in the test solution is 100-fold diluted. For recovery studies, known concentrations of GSH were added to the samples and the total thiol concentrations were then determined.

Cytotoxicity: The cell line, L929 (murine aneuploid fibro-sarcoma cell) was incubated in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) at 37°C with 5% CO_2. The cytotoxicity of PyT-Hg(II)-TPy and Hg^{2+} against L929 cells was assessed by MTT assay according to ISO 10993-5.



Scheme S1. Synthesis route for PyT.

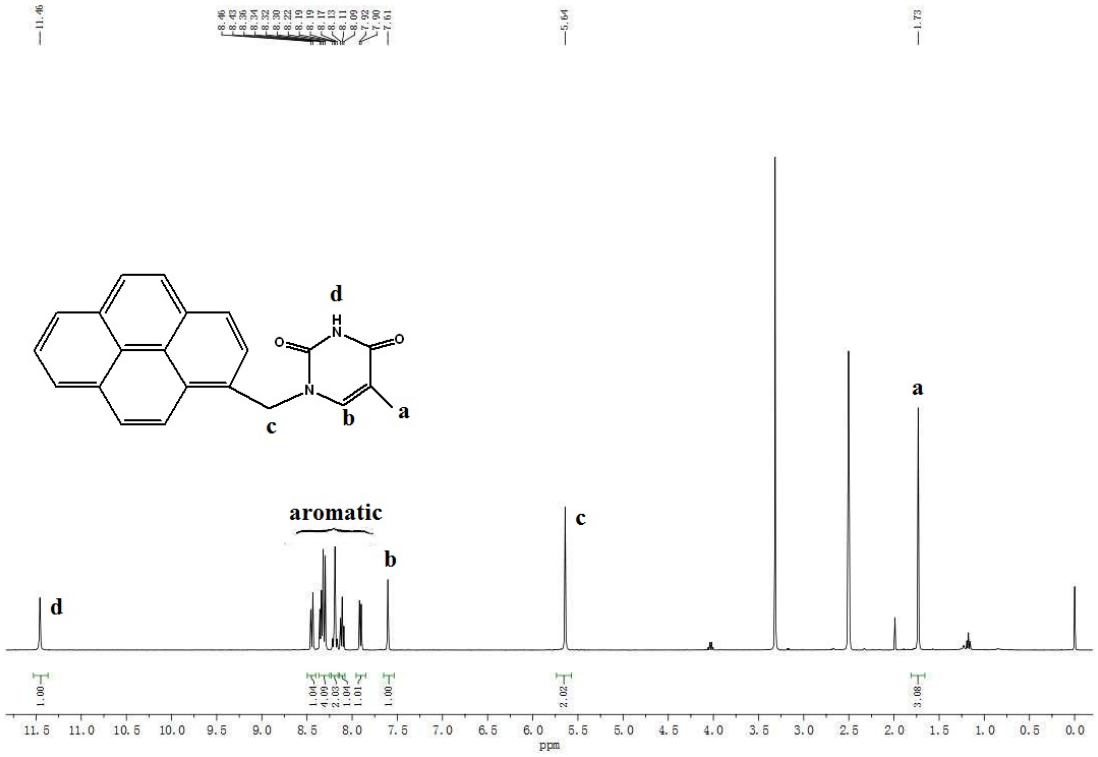


Fig. S1. ^{1}H NMR spectrum of PyT in DMSO-d_6.

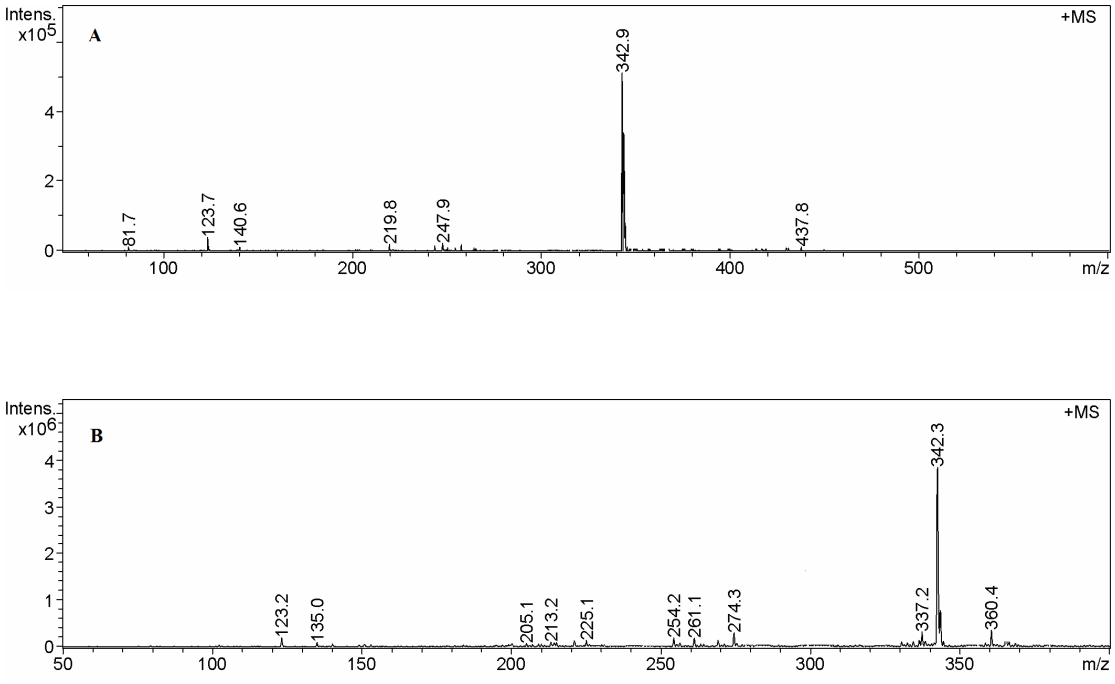


Fig. S2. Mass spectra of the as-prepared PyT (A) and the compound released from PyT-Hg(II)-TPy upon addition of GSH (B). For mass spectrum B, PyT-Hg(II)-TPy was dissolved in THF, excessive amount of GSH in water was added, after stirring for 10 min, THF was removed, the solid was washed with water for 3 times, and then used for measurement.

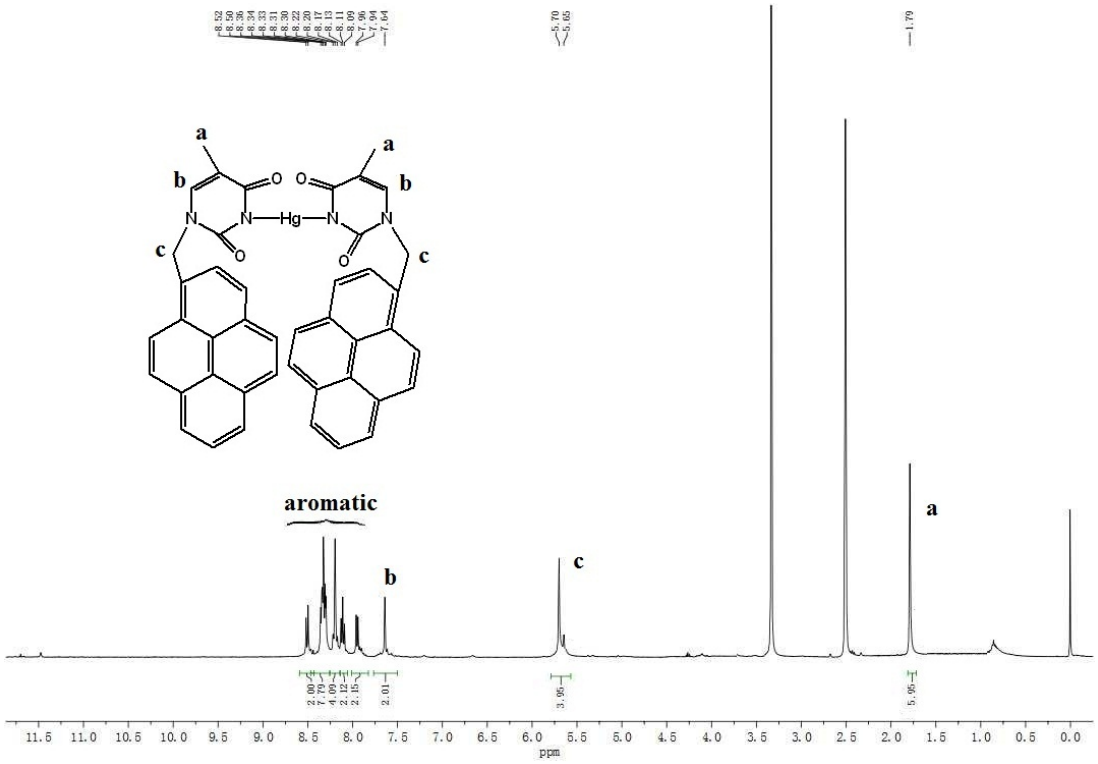


Fig. S3. ^{1}H NMR spectrum of PyT-Hg(II)-TPy in DMSO-d_6.

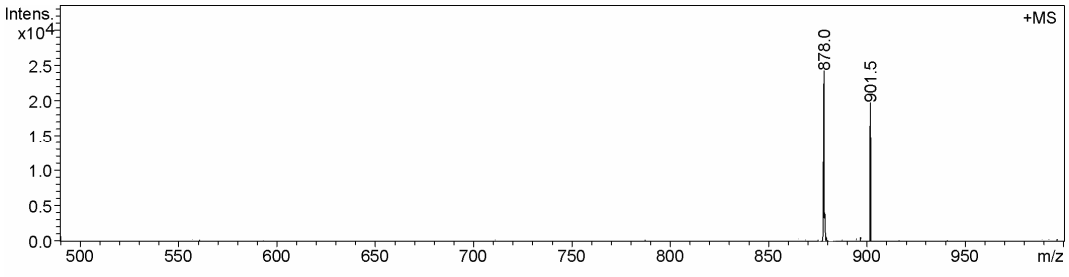


Fig. S4. Mass spectrum of PyT-Hg(II)-TPy.
m/z 878.0 [M^+], 901.5 [$M+Na^+$]

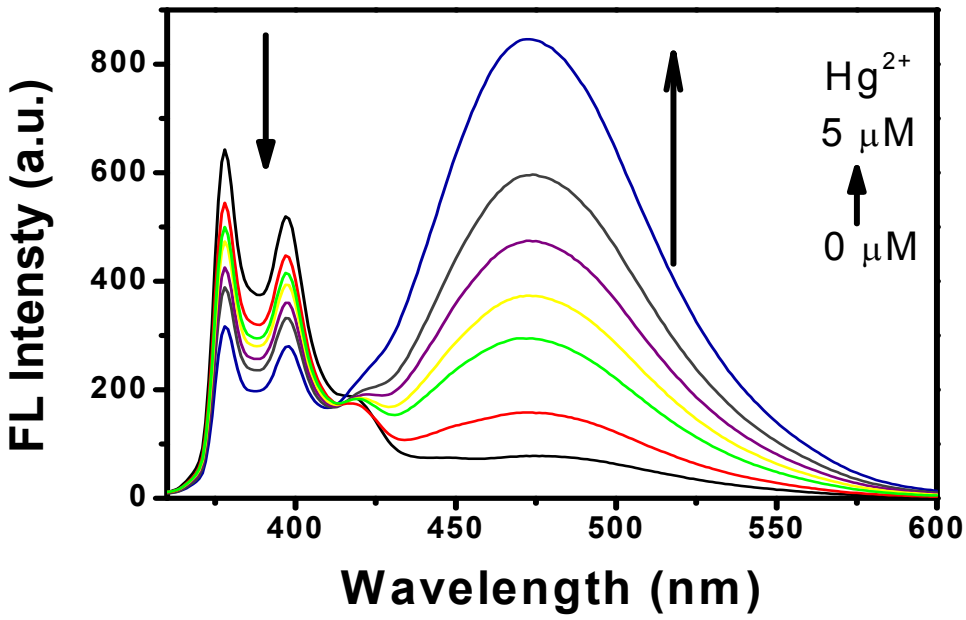


Fig. S5. Fluorescence spectra of PyT (1×10^{-5} M) in the presence of different amounts of Hg^{2+} in HEPES-buffered (pH 7.0) water-DMSO (7/1, v/v). Hg^{2+} concentration: 0, 1, 2, 2.5, 3, 4, 5 μM. (λexc = 345 nm).

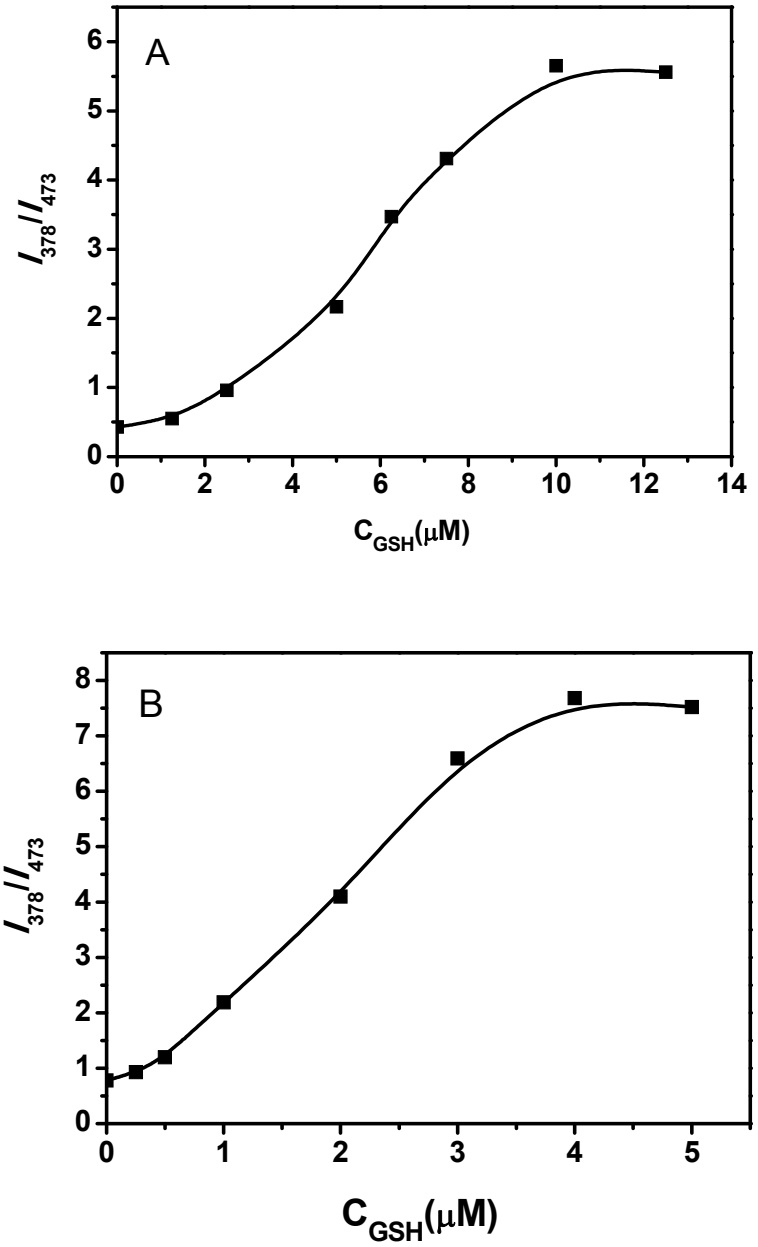


Fig. S6. Fluorescence intensity ratio of PyT-Hg(II)-TPy as a function of GSH concentration in HEPES buffered (pH 7.0) water-DMSO (7/1, v/v). (λexc = 345 nm). A: PyT-Hg(II)-Tpy concentration is 1×10^{-5} M; B: PyT-Hg(II)-Tpy concentration is 4×10^{-6} M.

Indeed the thiol calibration curve depends on the initial complex concentration. This would allow users to vary the calibration curve according to the concentrations they chose to some extent.

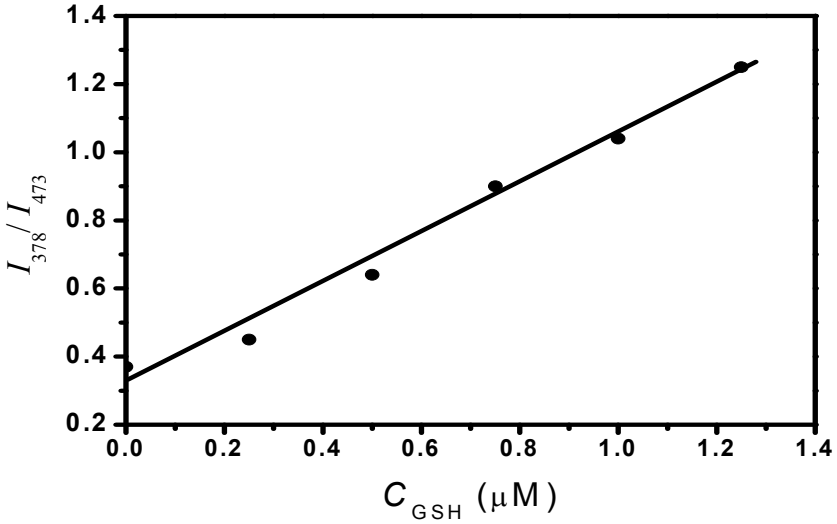


Fig. S7. The fluorescence intensity ratio of PyT-Hg(II)-TPy (5×10^{-6} M) as a function of GSH concentration in HEPES-buffered (pH 7.0) water-DMSO (7/1, v/v). GSH concentration: 0 - 1.25 μM (the lower concentration part). (λexc = 345 nm).

The method for determining the detection limit:

First the calibration curve was obtained from the plot of fluorescence intensity ratio, I_{378}/I_{473}, as a function of the analyte concentration (GSH). The regression curve equation was then obtained for the lower concentration part.

The detection limit = 3 × S.D./ k

where k is the slope of the curve equation, and S.D. represents the standard deviation for the PyT- Hg(II)-TPy solution intensity ratio in the absence of GSH.

I_{378}/I_{473} = 0.32 + 7.4 E5 [GSH] (R = 0.991)

LOD = 3 × 0.017 / 7.4 E5 = 69 nM

References:

V. Thomsen, D. Schatzlein, D. Mercuro, *Spectroscopy* **2003***, 18,* 112-114.

A. D. McNaught, A. Wilkinson, *IUPAC Compendium of Chemical Terminology,* **1997**.

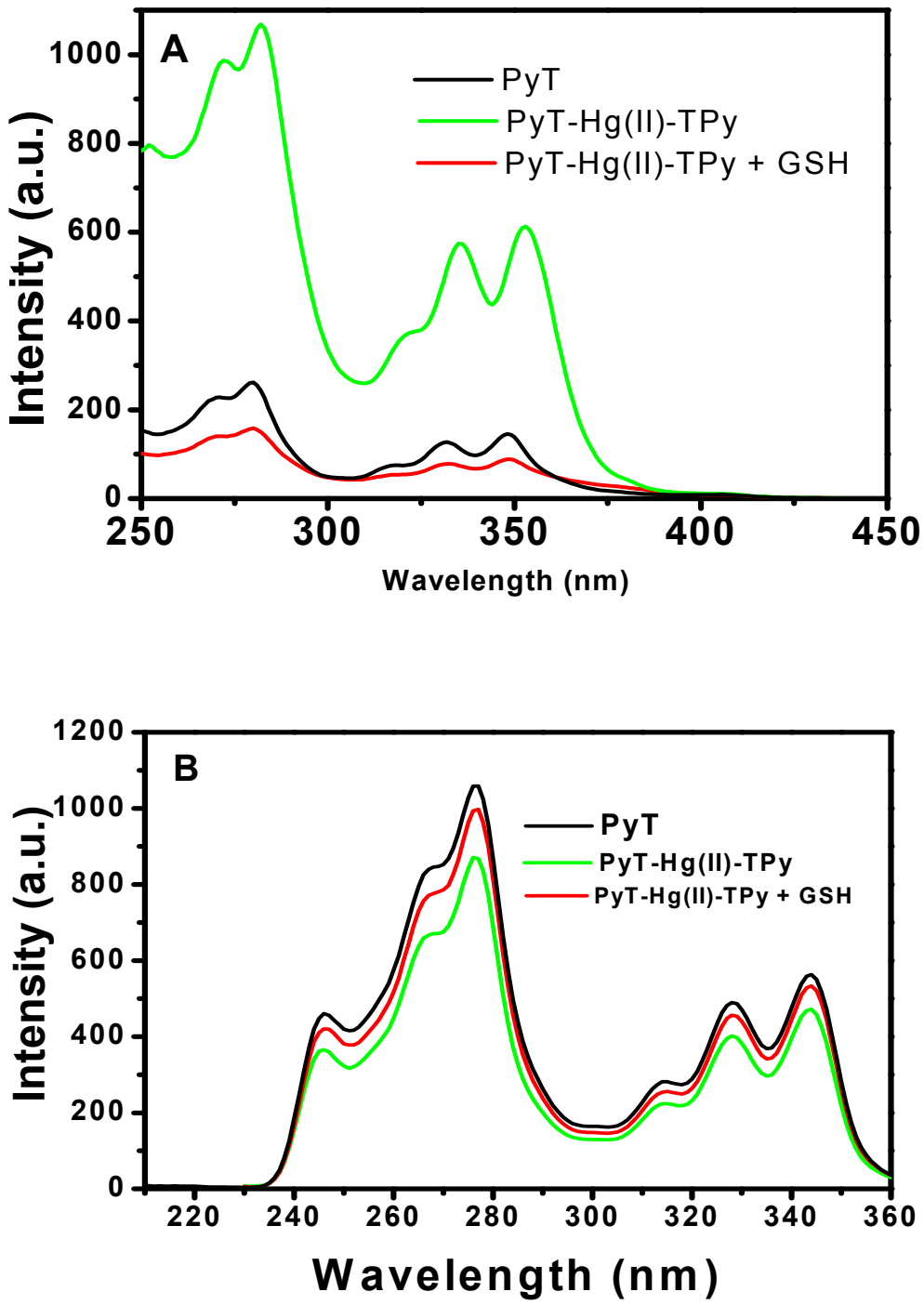


Fig. S8. The fluorescence excitation spectra of PyT (10 μM), PyT-Hg(II)-TPy (5 μM), and PyT-Hg(II)-TPy (5 μM) in the presence of GSH (5 μM) in HEPES-buffered (pH 7.0) water-DMSO (7/1, v/v). (A: λem = 473 nm, B: λem = 378 nm).

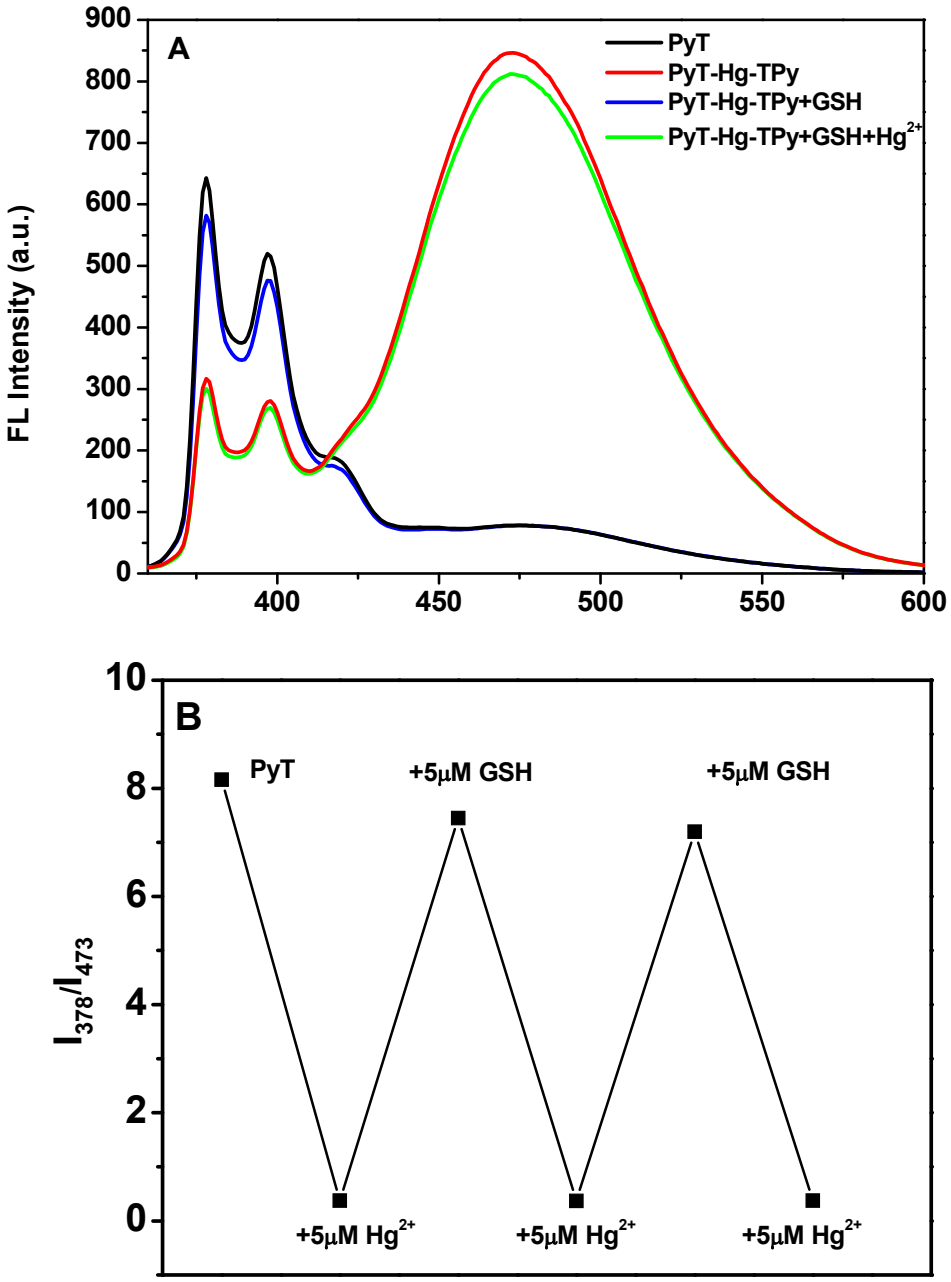


Fig. S9. Reversibility of fluorescence for PyT in HEPES buffered water/DMSO (7:1 v/v) upon alternate addition of mercury ions and GSH. (A) Fluorescence spectra. (B) Variation of fluorescence ratio.

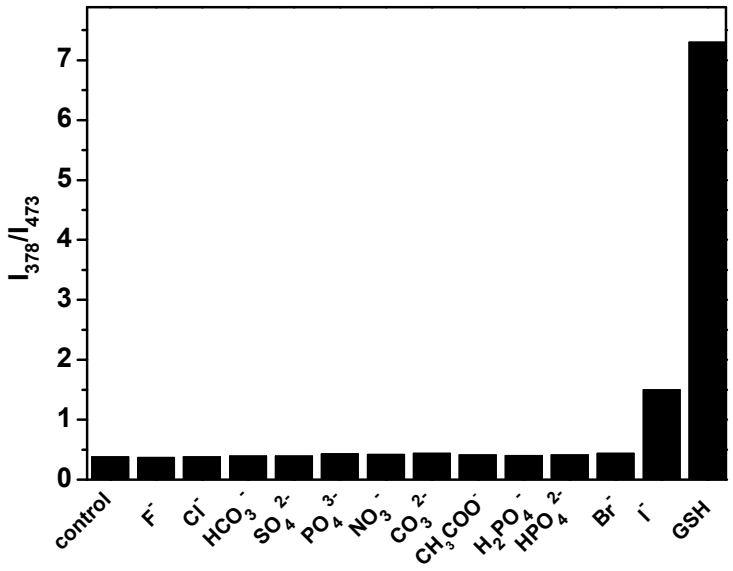


Fig. S10. Selectivity of PyT-Hg(II)-TPy (5×10^{-6} M) toward biological thiols over various anions (3 μM respectively) in HEPES buffered (pH 7.0) water-DMSO (7/1, v/v).

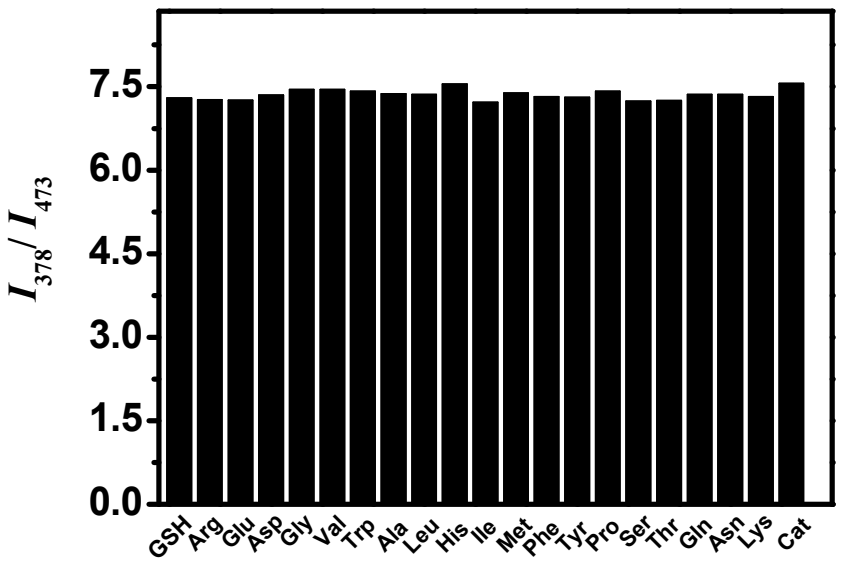


Fig. S11. Interference of amino acids and some biologically-abundant cations to PyT-Hg(II)-TPy (5 μM) for GSH (3 μM) sensing in HEPES-buffered (pH 7.0) water-DMSO (7/1, v/v). GSH: GSH only; Cat: GSH (3 μM) with Na^+ (10 mM)，K^+ (2 mM) and Ca^{2+} (2 mM) added; others are GSH (3 μM) with respective amino acid (100 μM) added. (λexc = 345 nm).

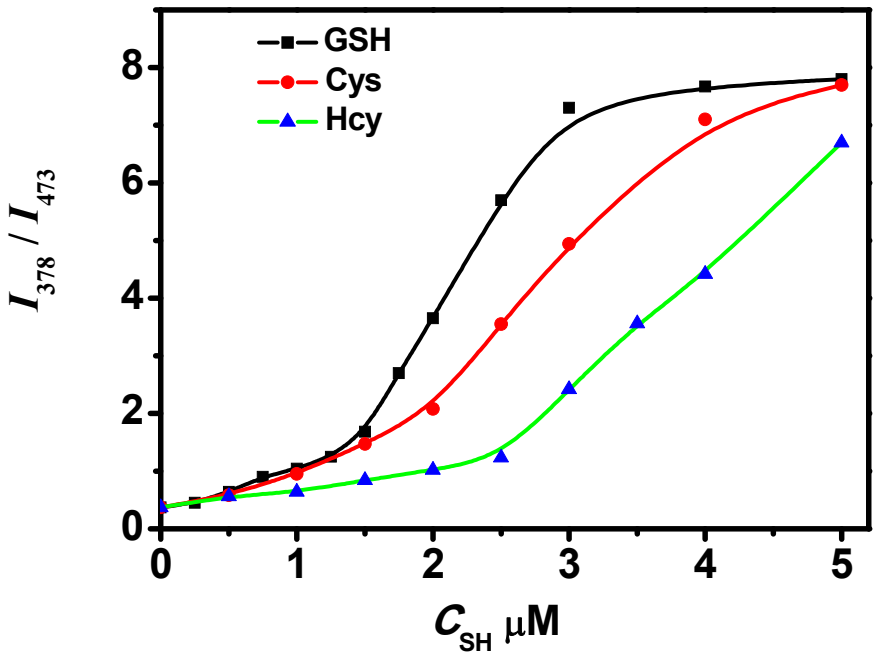


Fig. S12. The fluorescence intensity ratio of PyT-Hg(II)-TPy (5×10^{-6} M) as a function of thiols concentration in HEPES-buffered (pH 7.0) water-DMSO (7/1, v/v). (λexc = 345 nm). These curves can serve as the calibration curves.

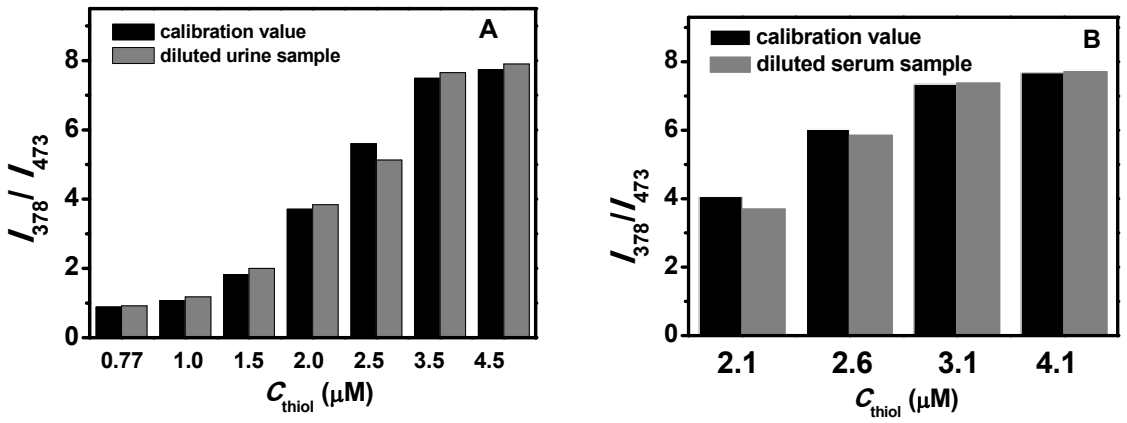


Fig. S13 (A). Comparison of fluorescence intensity ratio between that of PyT-Hg(II)-TPy (5 μM) in diluted urine (diluted by pH 7.0 HEPES buffered water-DMSO (7/1, v/v)) and that obtained from **GSH calibration curve** at different thiol concentrations (the orignailly-existing thiol plus spiked GSH). (λexc = 345 nm).
Black column: the fluorescence intensity ratio values obtained from the GSH calibration curve (as shown in Figure 2B) at different concentrations. The C_{thiol} concentrations represent the thiol (GSH) added into the HEPES buffered (pH 7.0) water-DMSO (7/1, v/v).
Grey column: the fluorescence intensity ratio for the diluted urine samples of different thiols concentrations (the determined original thiol concentration plus the varied spiked thiol concentrations). In the diluted urine sample without spiking GSH, it is determined that there are 0.5 μM thiols in the test solution (which means the concentration of thiols in the undiluted urine is ca. 25 μM). The C_{thiol} concentrations (0.77, 1.0, 1.5, 2.0, 2.5, 3.5, 4.5 μM) represent the total thiols concentration in the test samples, namely the 0.5 μM thiols plus the spiked GSH amounts (0.27, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0 μM).

(B). Comparison of fluorescence intensity ratio between that of PyT-Hg(II)-TPy (5 μM) in diluted serum (diluted by pH 7.0 HEPES buffered water-DMSO (7/1, v/v)) and that obtained from the **GSH calibration curve** at different thiol concentrations (the orignailly-existing thiol plus spiked GSH). (λexc = 345 nm).
Black column: the fluorescence intensity ratio values obtained from the GSH calibration curve (as shown in Figure 2B) at different concentrations. The C_{thiol} concentrations represent the thiol (GSH) added into the HEPES buffered (pH 7.0) water-DMSO (7/1, v/v).
Grey column: the fluorescence intensity ratio for the diluted serum samples of varied thiol concentrations (the determined original thiol concentration plus varied spiked thiol concentrations). In the diluted serum sample without spiking GSH, it is determined that there are 1.6 μM thiols in the test solution (which means the concentration of thiols in the undiluted serum is ca. 160 μM). The C_{thiol} concentrations (2.1, 2.6, 3.1, 4.1 μM) represent the total thiols concentration in the test samples, namely the 1.6 μM thiols plus the spiked GSH amounts (0.5, 1.0, 1.5, 2.5 μM).

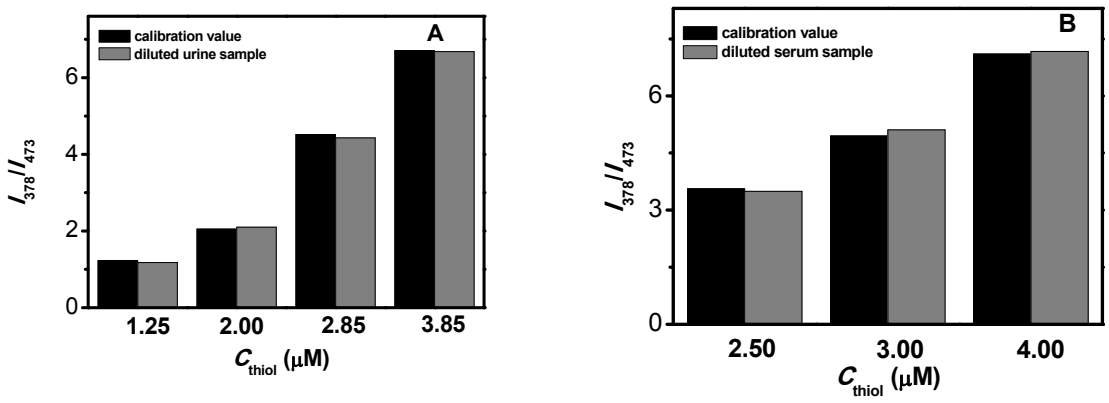


Fig. S14(A). Comparison of fluorescence intensity ratio between that of PyT-Hg(II)-TPy (5 μM) in diluted urine (diluted by pH 7.0 HEPES buffered water-DMSO (7/1, v/v)) and that obtained from **Cys calibration curve** at different thiol concentrations (the orignailly-existing thiol plus spiked Cys). (λexc = 345 nm).
Black column: the fluorescence intensity ratio values obtained from the Cys calibration curve (as shown in Figure S12) at different concentrations. The C_{thiol} concentrations represent the thiol (Cys) added into the HEPES buffered (pH 7.0) water-DMSO (7/1, v/v).
Grey column: the fluorescence intensity ratio for the diluted urine samples of different thiols concentrations (the determined original thiol concentration plus the varied spiked thiol concentrations). In the diluted urine sample without spiking Cys, it is determined that there are 0.5 μM thiols in the test solution (which means the concentration of thiols in the undiluted urine is ca. 25 μM). The C_{thiol} concentrations (1.25, 2.00, 2.85, 3.85 μM) represent the total thiols concentration in the test samples, namely the 0.5 μM thiols plus the spiked Cys amounts (0.75, 1.50, 2.35, 3.35 μM).

(B). Comparison of fluorescence intensity ratio between that of PyT-Hg(II)-TPy (5 μM) in diluted serum (diluted by pH 7.0 HEPES buffered water-DMSO (7/1, v/v)) and that obtained from the **Cys calibration curve** at different thiol concentrations (the orignailly-existing thiol plus spiked Cys). (λexc = 345 nm).
Black column: the fluorescence intensity ratio values obtained from the calibration curve (as shown in Figure 2B) at different concentrations. The C_{thiol} concentrations represent the thiol (Cys) added into the HEPES buffered (pH 7.0) water-DMSO (7/1, v/v).
Grey column: the fluorescence intensity ratio for the diluted serum samples of varied thiol concentrations (the determined original thiol concentration plus varied spiked thiol concentrations). In the diluted serum sample without spiking Cys, it is determined that there are 2.00 μM thiols in the test solution (which means the concentration of thiols in the undiluted serum is ca. 200 μM). The C_{thiol} concentrations (2.50, 3.00, 4.00 μM) represent the total thiols concentration in the test samples, namely the 2.0 μM thiols plus the spiked Cys amounts (0.50, 1.00, 2.00 μM).

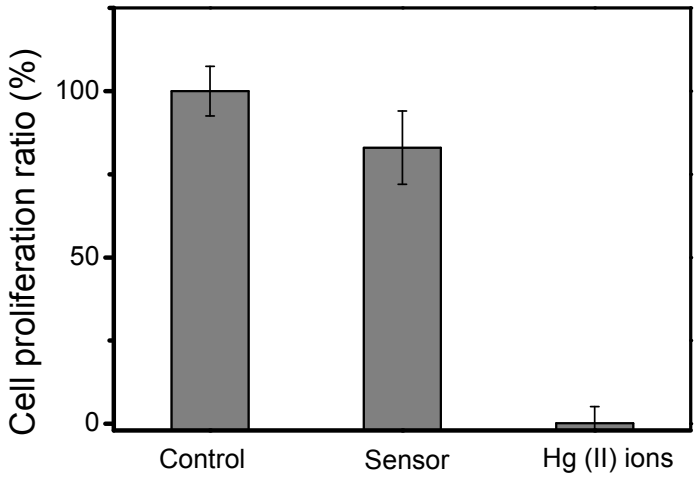


Fig. S15. Cytotoxic effects against L929 cells upon 24 hours of incubation. Control: L929 cells in the absence of the probe or mercury ions; Sensor: L929 cells in the presence of PyT-Hg(II)-TPy at the concentration of 5×10^{-6} M; Hg (II) ions: L929 cells in the presence of Hg^{2+} at the concentration of 5×10^{-6} M.

Note: Hg^{2+} is highly toxic, it is found that after two hours of cell culturing, almost all cells die. After 24 hours of incubation, the ion shows a Grade-IV toxicity, the highest grade according to United States Pharmacopoeia and ISO 10993-5 (Fig. S15); while the complex PyT-Hg(II)-TPy shows a Grade-I toxicity, indicating that the complex's toxicity is low.

Relationship between cell relative growth rate and cytotoxicity grade from United States Pharmacopeia (USP):

1. Grade 0 toxicity: cell proliferation ratio ≥ 100 %
2. Grade I toxicity: cell proliferation ratio ≥ 80 %
3. Grade II toxicity: cell proliferation ratio ≥ 50 %
4. Grade III toxicity: cell proliferation ratio ≥ 30 %
5. Grade IV toxicity: cell proliferation ratio ≥ 0 %

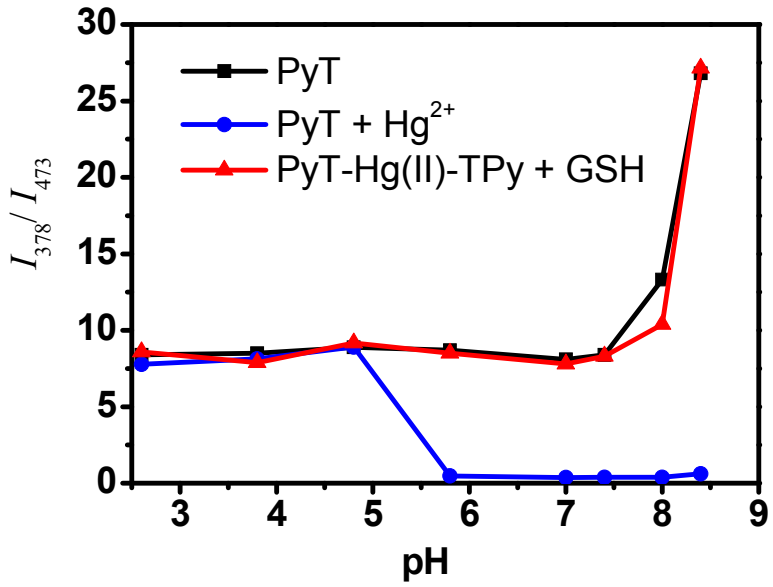


Fig. S16. Fluorescence intensity ratio of PyT (10 μM), PyT (10 μM) in the presence of Hg^{2+}(5 μM), and PyT-Hg(II)-TPy (5 μM) in the presence of GSH (5 μM) as a function of pH in HEPES-buffered (pH 7.0) water-DMSO (7/1, v/v). (λexc = 345 nm).

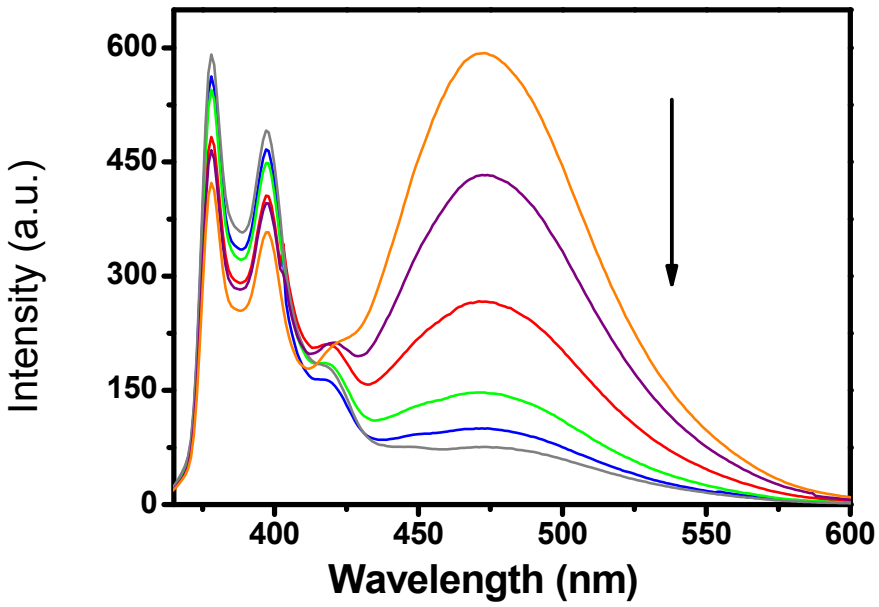


Fig. S17. Fluorescence spectra of diluted urine samples (50-fold diluted) with spiked GSH. As pointed by the arrow, from top to bottom, the spiked GSH levels are: 0 μM, 0.5 μM, 1.0 μM, 1.5 μM, 2.0 μM, 4.0 μM.

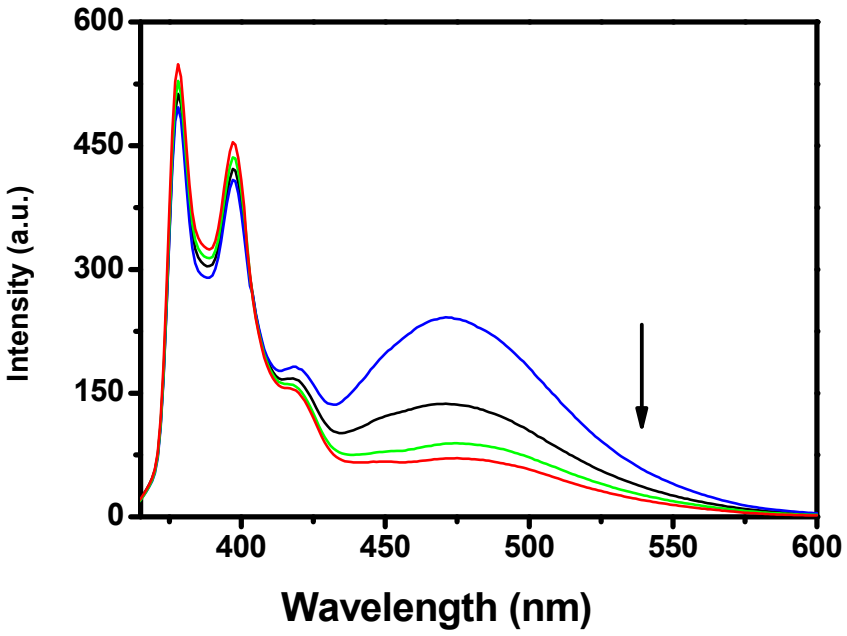


Fig. S18. Fluorescence spectra of diluted serum samples (100-fold diluted) with spiked GSH. As pointed by the arrow, from top to bottom, the spiked GSH levels are: 0 μM, 0.5 μM, 1.0 μM, 2.5 μM.

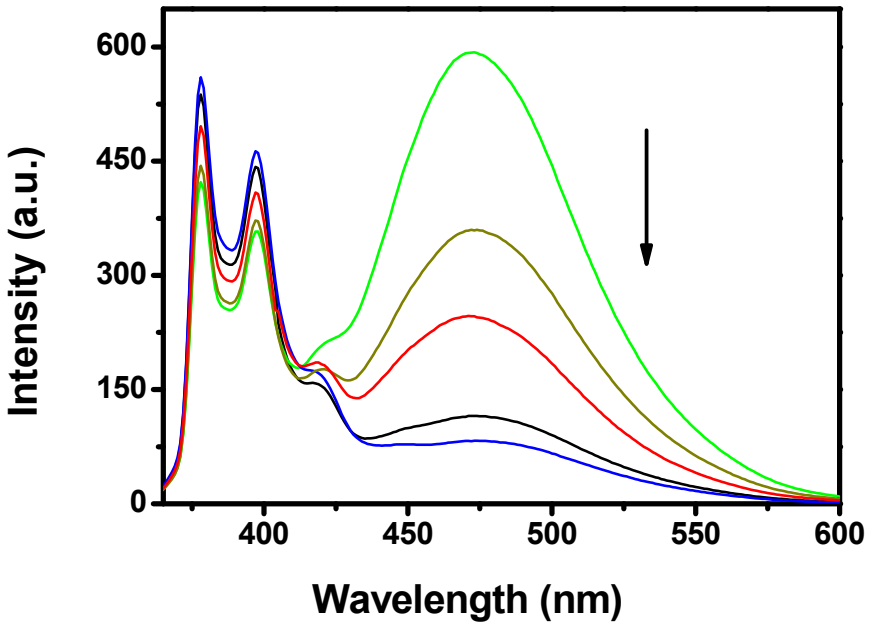


Fig. S19. Fluorescence spectra of diluted urine samples (50-fold diluted) with spiked Cys. As pointed by the arrow, from top to bottom, the spiked Cys levels are: 0 μM, 0.75 μM, 1.5 μM, 2.35 μM, 3.35 μM.

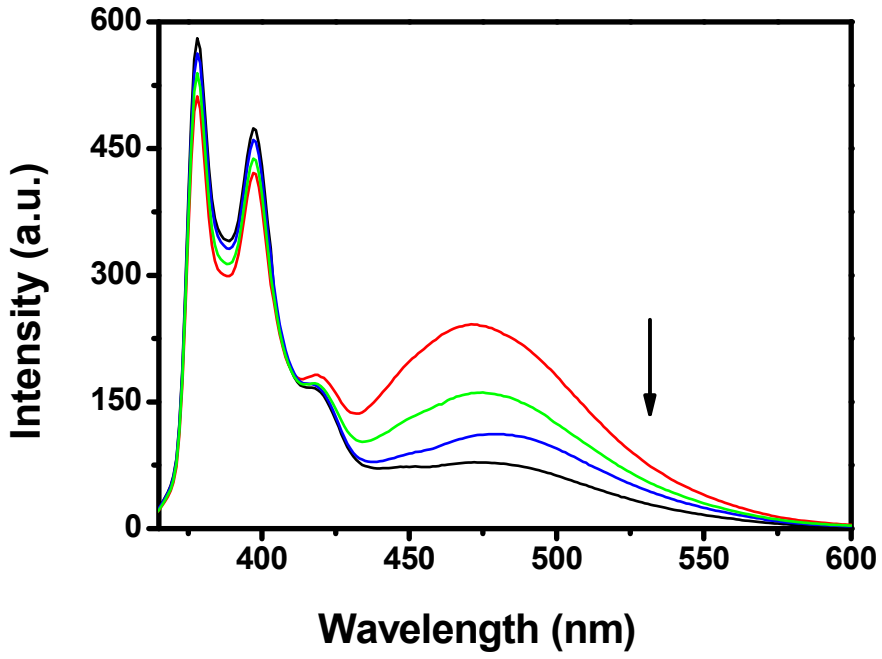


Fig. S20. Fluorescence spectra of diluted serum samples (100-fold diluted) with spiked Cys. As pointed by the arrow, from top to bottom, the spiked Cys levels are: 0 μM, 0.5 μM, 1.0 μM, 2.0 μM

Table S1. Determination of endogenous (originally-existing) thiols and that plus spiked GSH levels in human urine (50-fold diluted) using PyT-Hg(II)-TPy as the sensor.

Sample	Determined biothiol [(a)] (10^{-6} M)	Added GSH (10^{-6} M)	Combined thiol (10^{-6} M)	Measured (10^{-6} M)	Recovery (%)	RSD (n=3 ,%)
1	0.5	-	-	-	-	-
2		1.00	1.50	1.63	91.0	2.7
3		2.00	2.50	2.27	109.0	3.9
4		3.00	3.50	3.57	98.0	2.6
5		4.00	4.50	4.59	98.0	2.8

Note: (a) The determined biothiol value in the diluted urine sample without spiking GSH is 0.5 μM, **which means the biothiol concentration in undiluted urine sample is ca. 25 μM**. GSH calibration curve was used as the standard.

Table S2. Determination of endogenous (originally-existing) thiols and that plus spiked GSH levels in serum (100-fold diluted) using PyT-Hg(II)-TPy as the sensor.

Sample	Determined biothiol [(a)] (10^{-6} M)	Added GSH (10^{-6} M)	Combined thiol (10^{-6} M)	Measured (10^{-6} M)	Recovery (%)	RSD (n=3 ,%)
1	1.60	-	-	-	-	-
2		0.50	2.1	1.95	93.0	3.4
3		1.00	2.6	2.50	96.0	2.9
4		1.50	3.1	3.16	98.0	2.5
5		2.50	4.1	4.18	98.0	2.7

Note: (a) the determined biothiol value in the diluted serum sample without spiking GSH is 1.6 μM, **which means the biothiols concentration in undiluted serum is ca. 160 μM**. GSH calibration curve was used as the standard.

Appropriate dilution of urine and serum was necessary to ensure the concentration of biothiols in the preferable range and to obtain quantitative recovery of the spiked thiols (*Anal. Chem.* **2009,** *81,* 5001–5007).

Table S3. Determination of endogenous thiols and that plus spiked Cys levels in human urine (50-fold diluted) using PyT-Hg(II)-TPy as the sensor.

Sample	Determined biothiol [(a)] (10^{-6} M)	Added Cys (10^{-6} M)	Combined thiol (10^{-6} M)	Measured (10^{-6} M)	Recovery (%)	RSD (n=3 ,%)
1	0.5	-	-	-	-	-
2		0.75	1.25	1.20	96.3	3.5
3		1.50	2.00	2.05	102.5	3.9
4		2.35	2.85	2.79	97.9	2.3
5		3.35	3.85	3.84	99.7	2.1

Note: (a) The determined biothiol value in the diluted urine sample without spiking Cys is 0.5 μM, **which means the biothiol concentration in undiluted urine sample is ca. 25 μM**. Cys calibration curve was used as the standard.

Table S4. Determination of endogenous thiols and that plus spiked Cys levels in serum (100-fold diluted) using PyT-Hg(II)-TPy as the probe.

Sample	Determined biothiol [(a)] (10^{-6} M)	Added Cys (10^{-6} M)	Combined thiol (10^{-6} M)	Measured (10^{-6} M)	Recovery (%)	RSD (n=3 ,%)
1	2.00	-	-	-	-	-
2		0.50	2.50	2.45	98.0	3.6
3		1.00	3.00	3.07	102.3	2.5
4		2.00	4.00	4.04	101.0	2.2

Note: (a) the determined biothiol value in the diluted serum sample without spiking Cys is 2.0 μM, **which means the biothiols concentration in undiluted serum is ca. 200 μM**. Cys calibration curve was used as the standard.

The determined endogenous thiol concentration for the urine (25 μM) and serum (160 μM using GSH standard, or 200 μM using Cys standard) sample are within the normal range reported in previous literatures (S. Huang, Q. Xiao, R. Li, H. Guan, J. Liu, X. Liu, Z. He, Y. Liu, *Anal. Chim. Acta*, **2009**, 645, 73-78; C. Carru, A. Zinellu, S. Sotgia, R. Serra, M. F. Usai, G.F. Pintus, G.M. Pes, L. Deiana, 2004. *Biomed. Chromatogr.* 18, 360-366; J. V. Ros-Lis, B. Garci, D. Jimenez, R. Martinez-Manez, F. Sancenon, J. Soto, F. Gonzalvo, M.C. Valldecabres, **2004**. *J. Am. Chem. Soc.* 126, 4064-4065; B. Seiwert and U. Karst, *Anal. Chem.*, **2007**, 79, 7131; T. Ubuka, K. Kobayashi, K. Yao, H. Kodama, K. Fujii, K. Hirayama, T. Kuwari, S. Mizuhara, *Biochim. Biophys. Acta*, **1968**, 158, 493; S. Melnyk, M. Pogribna, I. Pogribny, R. J. Hine, S. J. James, *J. Nutr. Biochem.* **1999**, *10*, 490-497; W. G. Christen, U. A. Ajani, R. J. Glynn, C. H. Hennekens, *Arch. Intern. Med.*, **2000**, *160*, 422.)