Electronic Supplementary Information

A Dicopper Complex Chemiluminescence Probe for the Determination of Thiols in the Extracts of Murine P388 Lymphocytic Leukemia Cell

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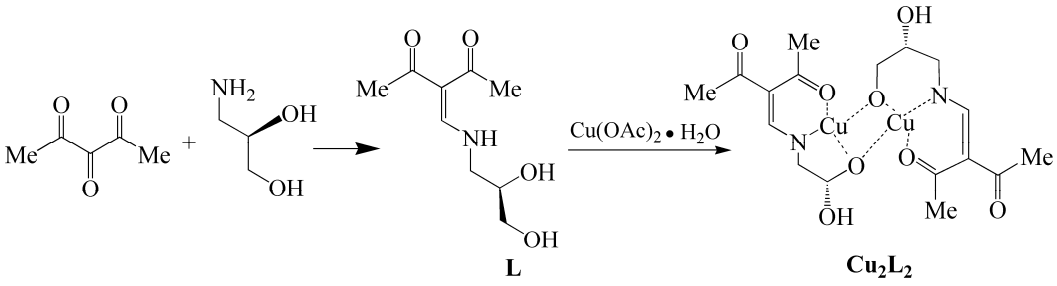
Synthesis of probe Cu_2L_2. Probe Cu_2L_2 was prepared and purified according to literature with some modifications.[1] Briefly, to solution of (R)-3-amino-1,2-propanediol (200 mg, 2.2 mmol) and 3-ethoxymethylene acetylacetone (514 mg, 3.3 mmol) in distilled methanol (20 mL), triethyl amine (458 μL, 3.3 mmol) was added and then stirred at room temperature (rt) under Ar atmosphere for 1 h. At the end of the reaction as judged by TLC analysis, the reaction solution was concentrated. The crude product was purified by silica gel column chromatography ($CHCl_3$: MeOH = 10: 1) to give colorless crystals (332 mg, 75 %). R_f = 0.37 ($CHCl_3$: MeOH = 10 : 1, *V* : *V*) , m.p. 87.0-88.5 ℃ (recrystallized from $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$): δ/ppm 2.01 (4H, br), 2.29-2.46 (4H, m), 3.42-3.47 (1H, m), 3.49-3.54 (1H, m), 3.64 (1H, dd, J = 5.5, 11.0 Hz), 3.71-3.77 (1H, m), 3.89-3.94 (1H, m), 7.80 (1H, d, J = 13.5 Hz), 11.08 (1H, br).

Triethylamine (415 μL, 3.0 mmol) was added into a solution of the obtained crystal (300 mg, 1.49 mmol) and $Cu(OAc)_2 \cdot H_2O$ (298 mg, 1.50 mmol) in distilled methanol (40 mL) and the reaction mixture was stirred at rt for 24 h. After evaporated to about 7 mL, the residue was diluted by diethylether (60 mL), and cooled in an ice bath. Pale blue precipitates were collected by filtration, and a fraction of the crude precipitates was recrystallized from toluene to give pale blue crystals (118 mg, 30 %). m.p. 215.4-216.5 ℃. ESI MS (negative mode): m/z: cacld for $C_{18}H_{26}Cu_2N_2O_8$ ([M - H]$^-$), 523.0, 525.0, found 523.1, 525.1.



Schem S1. Synthesis of probe Cu_2L_2

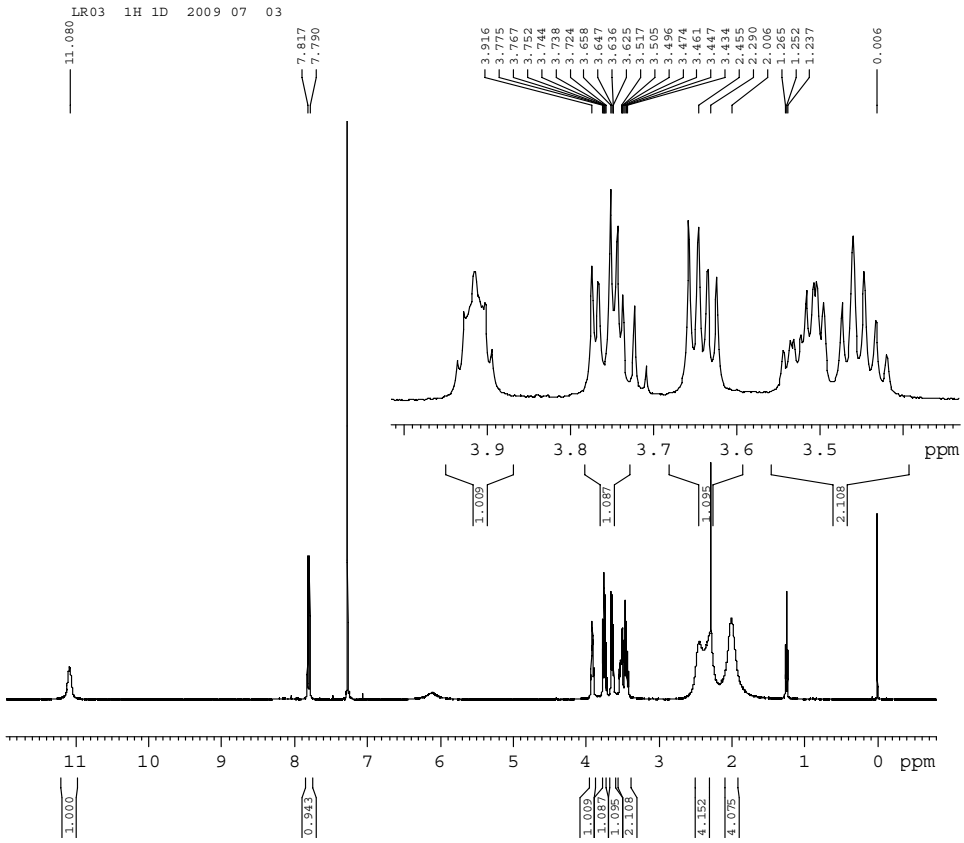


Fig. S1 ^{1}H NMR spectrum of compound **L**

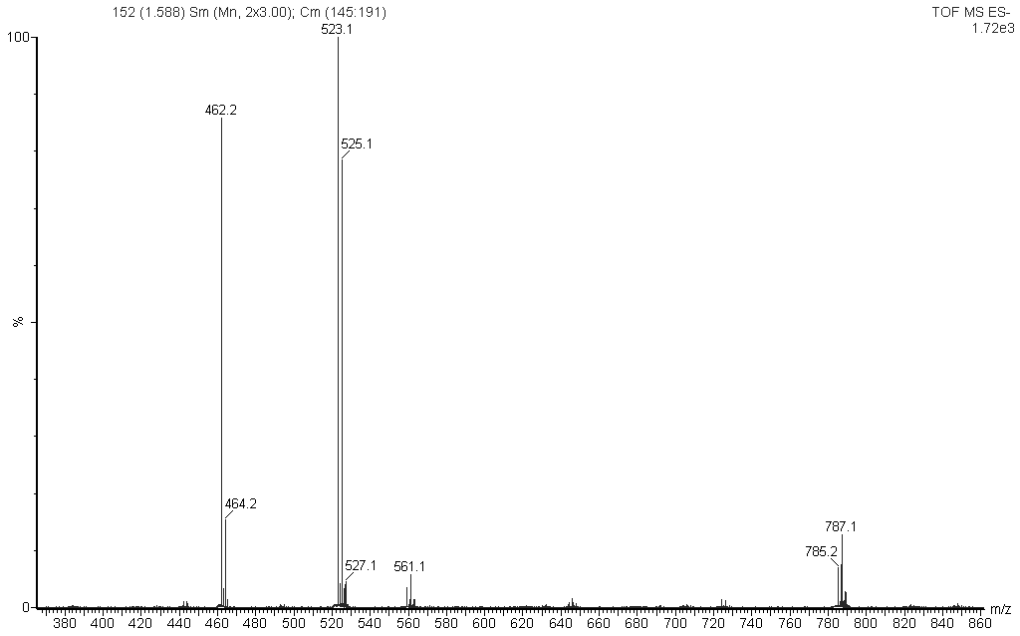


Fig. S2 MS spectrum of compound Cu_2L_2

Catalytic effect of synthesized probe Cu_2L_2 and Cu^{2+} for luminol chemiluminescence

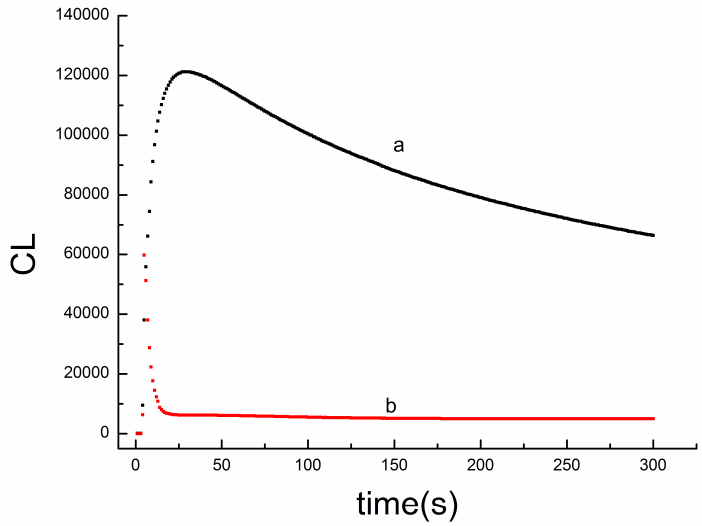


Fig. S3. CL responses of luminol (1.5×10^{-4} M)-H_2O_2 (2.0×10^{-2} M) catalyzed by (a)probe Cu_2L_2 (2.0 μM); (b) Cu^{2+}(2.0 μM) at pH 10.0.

Apparatus. The CL measurements were performed with a BPCL ultraweak luminescence analyzer (Institute of Biophysics Academic Sinica, China). UV-Visible spectra were carried out on a Cary 50 UV-Vis-NIR spectrophotometer (Varian). The pH value was measured with a PHS-3D pH-meter (Shanghai Lei Ci Device Works, China) with a combined glass-calomel electrode. ICP-MS experiments were performed on Agilent 7500a series.

Materials. Unless otherwise specified, all reagents were of analytical reagent grade and all solutions were prepared with double-distilled water. Glutathione (GSH), Dithiothreitol (DTT), thioglycolic acid (TA), 2-mercaptoethanol (β-ME), cysteine (Cys), glucose, alanine, cholesterol, ascorbic acid, L-phenylalanine, dopamine hydrochloride, leucine, uric acid, epinephrine were all purchased from Sigma Chemical Co. (St. Louis, MO, USA). Glutathione reductase (GR) (from yeast, 1000 U/mL) was purchased from Shanghai Sangon Biological Engineering Technology &

Services Co. Ltd. (China). Zinc metallothionein (MT) (from rabbit liver) was purchased from Hunan Lugu Biotechnology Co. Ltd. (China).

Stock solution of Probe $Cu_2L_2$was prepared by dissolving 16 mg probe Cu_2L_2 in 10 mL water, giving a molar concentration of 3.0×10^{-3} M. A 10 mM luminol stock solution was prepared by dissolving 177.2 mg luminol in 100 mL 0.1 M NaOH solution. A working solution of luminol was prepared by diluting the stock with 100 mM H_3BO_3-NaOH buffer solution. A stock solution of 2.0×10^{-2} M H_2O_2 was prepared by diluting 205 μL 30% (v/v) H_2O_2 to 100 mL with water and titrating with standard potassium permanganate, and working solutions of H_2O_2 were prepared by further dilution of this stock solution. The stock solution of 1.0×10^{-2} M GSH was prepared freshly by dissolving 32 mg GSH in 100 mL water and stored in a refrigerator at 4°C to avoid exposure to light and air.

Reaction of GSH with probe Cu_2L_2 and CL measurement. Solution of probe Cu_2L_2 (1.0×10^{-4} M, 100 μL) and GSH were placed in a 10 mL calibrated tube, then diluted with H_3BO_3-NaOH buffer (pH 6.5) to 5 mL. After vortex mixing at rt for 10 min, the result solution was submitted to CL measurement.

Reaction was initiated by the addition of H_2O_2 (2.0×10^{-2} M, 25 μL) into a reaction mixture (pH 10.0) which contained luminol sodium (1.5×10^{-4} M in 0.1 M H_3BO_3-NaOH buffer, 350 μL), 100 μL probe Cu_2L_2 before and after treated with GSH obtained above and 100 mM H_3BO_3-NaOH buffer (105 μL). Luminescence was measured by a luminometer and the CL produced was measured for 300 s. The total CL intensity was defined as the area under the CL decay curve.

Conditions between the reaction of probe Cu_2L_2 and GSH. H_3BO_3-NaOH was selected as the buffer solution in our experiments according to the literature.[1] The pH value has a great effect on the reaction of probe Cu_2L_2 with GSH. Because of the instability of dicopper complex in strong acid condition, the use of strongly acidic solution was avoided. Moreover, copper (II) complex will deposit in alkali solution. Thus the optimal pH values of the system were studied in the range 5.0-10.0. As shown in Fig. S4, the decrease of CL intensity (ΔI_{CL}) increases when the pH ranges from 5.0 to 6.5 and then decreases gradually since 6.5. Therefore, the H_3BO_3-NaOH buffer with pH 6.5 was selected in the competition reaction.

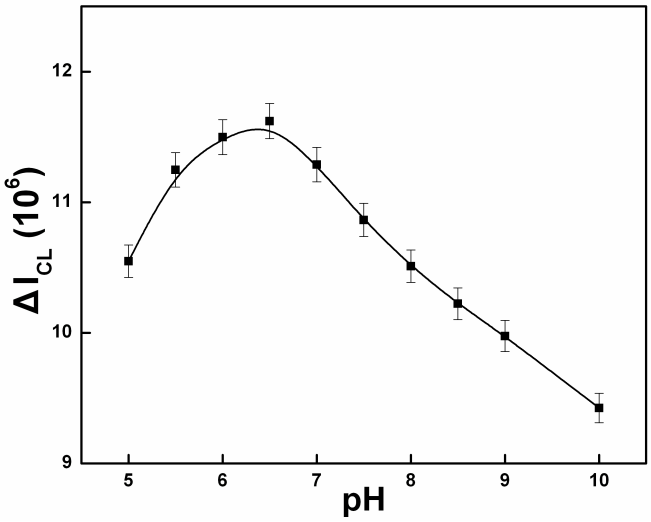


Fig. S4. Effect of pH values. Condition: Luminol, 1.5×10^{-4} M; H_2O_2, 2.0×10^{-2} M; Cu_2L_2, 2.0×10^{-6} M; GSH, 1.0×10^{-7} M, H_3BO_3-NaOH buffer solution (0.1 M); CL measurement was performed at pH 10.0.

Reaction time is also an important factor in optimizing experimental variables and the time required for reaction of probe Cu_2L_2 with GSH at 25ºC was investigated. As shown in Fig. S5, the ΔI_{CL} reached a maximum after the reagents had been added for ~10 min and remained constant for at least 40 min. Hence, the reaction was left to proceed for 10 min.

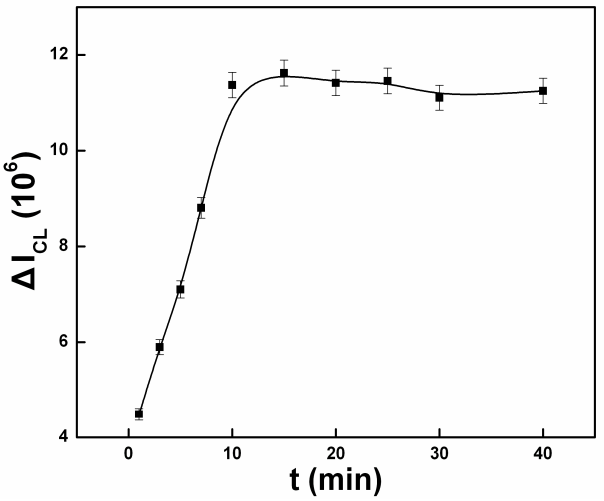


Fig. S5 Effect of reaction time. Condition: Luminol, 1.5×10^{-4} M; H_2O_2, 2.0×10^{-2} M; Cu_2L_2, 2.0×10^{-6} M; GSH, 1.0×10^{-7} M, H_3BO_3-NaOH buffer solution (0.1 M); Reaction of GSH with Cu_2L_2 was performed at pH 6.5; CL measurement was performed at pH 10.0.

Conditions of CL measurement. The effect of the pH on ΔI_{CL} during CL measurement was studied in the pH range 8.5-12.0. Fig. S6 shows that ΔI_{CL} increase from pH 8.5 to 10.0, and the maximum ΔI_{CL} was obtained at pH 10.0. Therefore, 0.1 M H_3BO_3-NaOH buffer with pH 10.0 was selected for the best CL measurement response in following experiments.

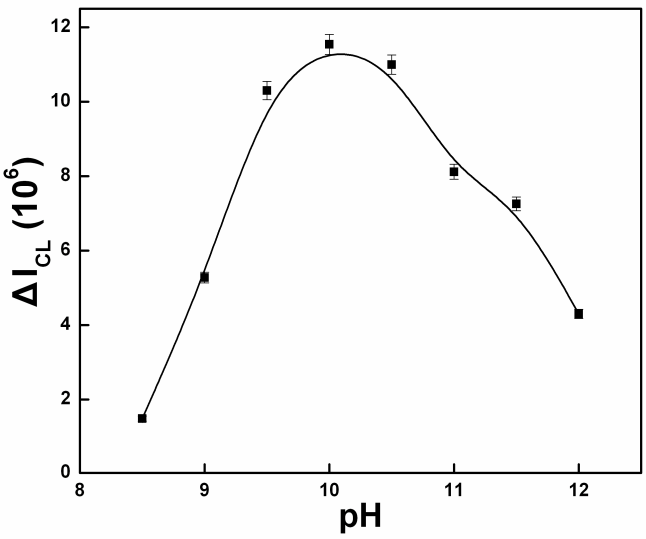


Fig. S6. Influence of pH values. Condition: Luminol, 1.5×10^{-4} M; H_2O_2, 2.0×10^{-2} M; Cu_2L_2, 2.0×10^{-6} M; GSH, 1.0×10^{-7} M, H_3BO_3-NaOH buffer solution (0.1 M); reaction of GSH with Cu_2L_2 was performed at pH 6.5.

The effect of luminol concentration on ΔI_{CL} was examined from 5.0×10^{-5}-1.2×10^{-3} M. As shown in Fig. S7, the maximum CL intensity was obtained when the luminol concentration was 1.5×10^{-4} M, then ΔI_{CL} was reduced gradually. Therefore, 1.5×10^{-4} M luminol was used as an optimum concentration.

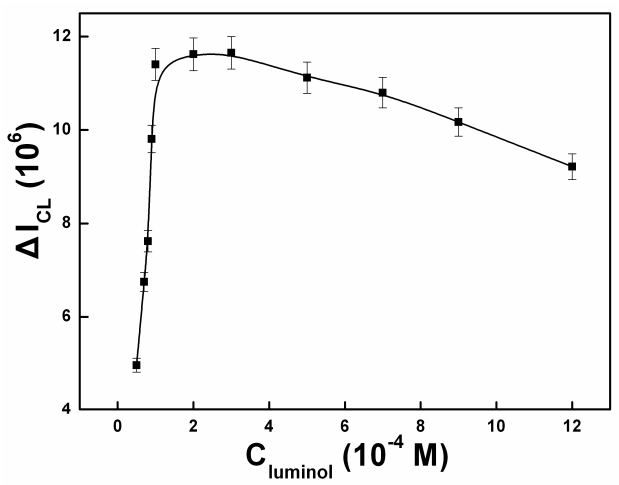


Fig. S7. Effect of luminol concentrations. Condition: H_2O_2, 2.0×10^{-2} M; Cu_2L_2, 2.0×10^{-6} M; GSH, 1.0×10^{-7} M, H_3BO_3-NaOH buffer solution (0.1 M); Reaction of GSH with Cu_2L_2 was performed at pH 6.5; CL measurement was performed at pH 10.0.

The effect of hydrogen peroxide concentrations on the CL reaction was examined in the range of 5.0×10^{-3}-1.2×10^{-1} M. As shown in Fig. S8, ΔI_{CL} increased with increasing concentration of H_2O_2 in the range 5.0×10^{-3}- 2.0×10^{-2} M. Over 2.0×10^{-2} M, ΔI_{CL} decreased gradually. So the concentration 2.0×10^{-2} M hydrogen peroxide was chosen for the optimal concentration in this study.

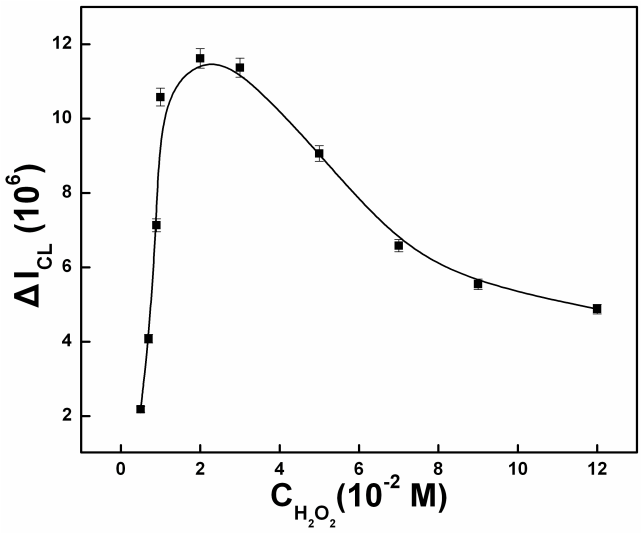


Fig. S8. Effect of hydrogen peroxide concentration. Condition: Luminol, 1.5×10^{-4} M; Cu_2L_2, 2.0×10^{-6} M; GSH, 1.0×10^{-7} M, H_3BO_3-NaOH buffer solution (0.1 M); Reaction of GSH with Cu_2L_2 was performed at pH 6.5; CL measurement was performed at pH 10.0.

Fig. S9showed that ΔI_{CL} increased linearly with the increasement of probe Cu_2L_2 concentration from 2.0×10^{-6}-7.0×10^{-6} M. Considering the CL intensity and the consumption of the reagents, the optimized concentration of probe Cu_2L_2 was 2.0×10^{-6} M.

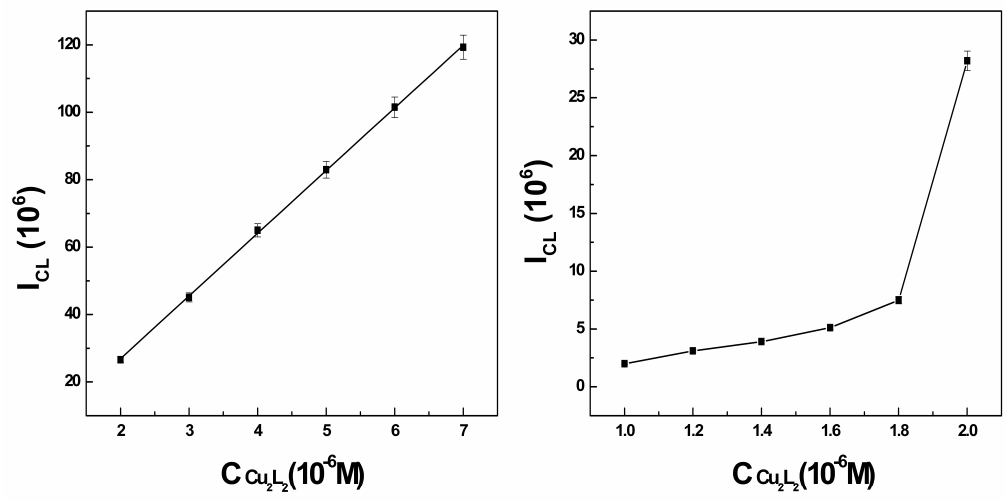


Fig. S9. Effect of dicopper complex concentration on CL intensity. Condition: Luminol, 1.5×10^{-4} M; H_2O_2, 2.0×10^{-2} M; H_3BO_3-NaOH buffer solution (0.1 M); CL measurement was performed at pH 10.0.

Interferences of various coexisting non-thiols substances

Ten kinds of metal ions and nine kinds of non-thiol compounds were tested to investigate the

selectivity of present method. The tolerance limit of interference was taken as a relative error not greater than ± 5% for 2.0×10^{-8} M GSH. The results were shown in Fig. 3 of manuscript, and detail data were shown in Table S1.

Table S1. Interferences of various coexisting non-thiols substances

Coexisting substance	Concentration (mol/L)	Relative error (%)	Coexisting substance	Concentration (mol/L)	Relative error (%)
Na^+	4×10^{-4} (2×10^{-4})	4.1	Cholesterol	8×10^{-5} (3000)	-4.5
K^+	4×10^{-4} (2×10^{-4})	2.9	Ascorbic acid	4×10^{-5} (2000)	2.8
Ca^{2+}	8×10^{-5} (4000)	4.7	L-Phenylalanine	1×10^{-5} (500)	-4.8
NH_4^+	8×10^{-5} (4000)	-4.2	Leucine	8×10^{-6} (400)	-3.7
Mg^{2+}	1×10^{-5} (500)	3.8	Epinephrine	8×10^{-6} (400)	-2.7
Zn^{2+}	1×10^{-5} (500)	3.2	Dopamine	6×10^{-6} (300)	-4.6
Cu^{2+}	1×10^{-6} (50)	4.6	Uric acid	6×10^{-6} (300)	-3.3
Fe^{3+}	1×10^{-6} (50)	3.5	Glucose	2×10^{-4}(1×10^{4})	2.5
Co^{2+}	4×10^{-7} (20)	4.5	Alanine	2×10^{-4}(1×10^{4})	-4.3
Ni^{2+}	4×10^{-7} (20)	3.9			

Condition: Luminol, 1.5×10^{-4} M, H_2O_2, 2.0×10^{-2} M, Cu_2L_2, 2.0 μM; GSH, 2.0×10^{-8} M; Reaction of **Cu_2L_2** with GSH was performed at 0.1 M H_3BO_3-NaOH buffer solution (pH 6.5); CL measurement was performed at H_3BO_3-NaOH solution (pH 10.0).

Reactivity of Cu_2L_2 with two protein thiols

Two protein thiols of metallothionein (MT) and glutathione reductase (GR) were further testified as described in manuscript, and the results were shown in Fig. S10.

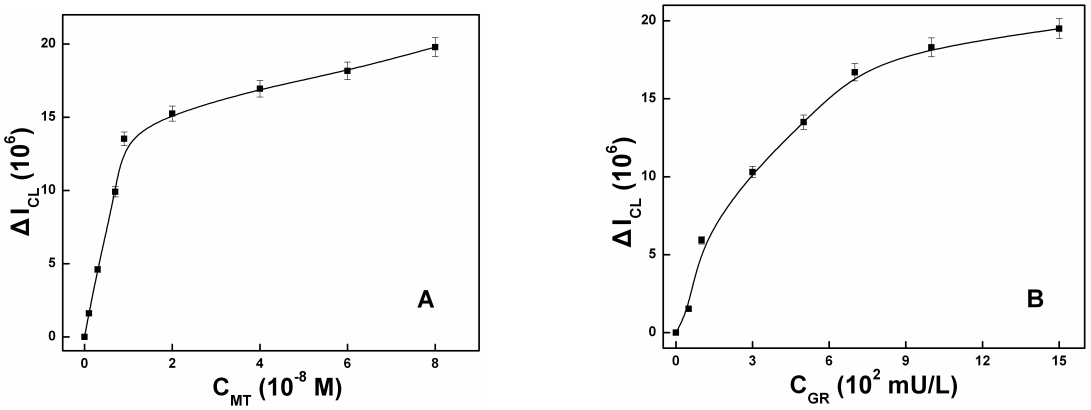


Fig. S10. CL responses toward (A): MT (1.0, 3.0, 7.0, 9.0, 20, 40, 60, 80 nM); (B): GR (50, 100, 300, 500, 700, 1000, 1500 mU/L). Other conditions were the same as those described in Table S1.

Detection of thiols in cell extracts

Murine P388 lymphocytic leukemia cell was counted at a density of 1.5×10^6 cells/mL for thiols detection. Cell extracts experiments were performed mainly according to methods reported by Choon-Nam Ong et al[2] with some modifications.

Detection of free thiols in cell extracts

Cells were firstly washed with ice-cold PBS (pH 7.4, 0.1 M) for three times and resuspended in 3 mL sodium phosphate buffer (PBS, pH 7.4, 0.1 M). After homogenization, the 3% perchloric acid was added into the cell homogenate to precipitate protein. With the mixture centrifuged at 14,000 rpm for 5 min at 4°C, the supernatant was collected for detection of free thiols. After a 10-fold dilution, aliquots (each 100 μL supernatant) were mixed with probe Cu_2L_2. The reaction and CL detection processes were carried out as described in experimental section.

Detection of protein thiols in cell extracts

Cells were washed with ice-cold PBS (pH 7.4, 0.10 M) and resuspended in protein precipitation solution (5% trichloroacetic acid (TCA)). The protein pellet was collected by centrifugation and washed with TCA solution. Then the protein was redissolved in 3 mL Tris-HCl buffer (pH 7.4, 0.10 M) containing 0.5% sodium laurylsulfonate (SDS). After a 10-fold dilution, aliquots (each 100 μL supernatant) were mixed with probe Cu_2L_2. The reaction and CL detection processes were carried out as described in experimental section.

The detection results of thiols in cell extracts were shown in Fig. 5 of manuscript. When total concentration of thiols in cell extracts was count by GSH and the GSH content of the cell extracts was derived from the standard curve and the regression equation, the concentration of deluted free thiols extracts is 7.4×10^{-8} M (3.7×10^{-5} M before dilution), and the concentration

of deluted protein thiols is 12.7×10^{-8} M (6.3×10^{-5} M before dilution).

Determination of thiols in cell extracts using proposed method and electrochemical method.

Non-protein cell extracts obtained as described above. After a 20-fold dilution, aliquots (each 50 μL supernatant) were mixed with probe Cu_2L_2 (1.0 × 10^{-4} M, 100 μL), then diluted with H_3BO_3-NaOH buffer (pH 6.5) to 5 mL. The reaction and CL detection processes were carried out as described in section of **Reaction of GSH with probe Cu_2L_2 and CL measurement** in ESI.

Protein cell extracts obtained as described above. After a 30-fold dilution, aliquots (each 50 μL supernatant) were mixed with probe Cu_2L_2 (1.0 × 10^{-4} M, 100 μL), then diluted with H_3BO_3-NaOH buffer (pH 6.5) to 5 mL. The reaction and CL detection processes were carried out as described in section of **Reaction of GSH with probe Cu_2L_2 and CL measurement** in ESI.

The total concentration of thiols in cell extracts was count by GSH and the GSH content of the cell extracts was derived from the standard curve and the regression equation. Electrochemical detection was carried out by our group using method reported previously.[3]

Table S2. Determination of thiols in cell extracts Using proposed method and electrochemical method[a]

Cell extracts	GSH added ($\times10^{-8}$, M)	Proposed method ($\times10^{-8}$, M)	cell extract thiols ($\times 10^{-5}$, M)	Electro-chemical method ($\times10^{-8}$ M)	cell extract thiols ($\times 10^{-5}$, M)	Recovery (%)	relative deviation (%)	$t_{exp.}$	$t_{tab.(95\%)}$
Non-protein extracts	0	1.85 (± 0.08)	3.70	1.94 (± 0.10)	3.88	-	-4.64	1.92	2.31
	2.0	3.94	-	-	-	104.5	-	-	-
	3.0	4.75	-	-	-	96.7	-	-	-
	4.0	5.92	-	-	-	101.8	-	-	-
	5.0	7.11	-	-	-	105.2	-	-	-
Protein extracts	0	2.06 (± 0.11)	6.18	2.11 (± 0.13)	6.33	-	-2.37	1.21	2.31
	2.0	4.13	-		-	103.5	-	-	-
	3.0	5.02	-		-	98.7	-	-	-
	4.0	6.17	-		-	102.8	-	-	-
	5.0	6.98	-		-	98.4	-	-	-

[a]Each value is the average of three measurements, the total concentration of thiols was count by GSH.

Influence of four metal ions

Meal ions such as Cu^{2+}, Fe^{3+}, Co^{2+} and Ni^{2+} can influence the CL effect. As shown from Table S1, when the concentrations of above metal ions were 50 or 20 times more than that of GSH, the interference was obtained. But compared with the trace levels of the metal ions in a cell extracts (as shown in Table S3), we can see that these four metal ions can not influence the detection of thiols in cell extracts.

Table S3 Concentration of thiols and metal ion[a] in cell extracts

Cell extracts	cell extracts thiols (M)[b]	Cu^{2+} (M)[c]	Fe^{3+} (M)[c]	Co^{2+} (M)[c]	Ni^{2+} (M)[c]
non-protein extracts	3.70×10^{-5}	2.27×10^{-6}	1.25×10^{-5}	3.08×10^{-6}	2.91×10^{-5}
protein extracts	6.18×10^{-5}	1.32×10^{-6}	7.38×10^{-6}	2.58×10^{-6}	2.66×10^{-6}

[a]Each value is the average of three measurements. [b]the concentration of thiols in the cell extracts was measured by our CL method, the total concentration of thiols was count by GSH; [c]The concentration of M^+ in the cell extracts was measured by ICP-MS.

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(3) Wang, W.; Li L.; Liu, S. F.; Ma, C. P.; Zhang, S. S. J. Am. Chem. Soc. 2008, 130, 10846-10847.