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In vivo monitoring of glutathione in a live rat brain based on the ratiometric signal output of 2D Cu-TCPP(Fe) nanosheets

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Experimental Section

Reagents and Chemicals. Fe(III) tetra(4-carboxyphenyl)porphine chloride (TCPP(Fe)) and tetrakis(4-carboxyphenyl) porphyrin (TCPP) were supplied by Frontier Scientific (Logan, Utah, USA). Trifluoroacetic acid, ascorbic acid (AA), glucose, uric acid (UA) and dopamine (DA) were purchased from Bidepharm Technology Co., Ltd. (Shanghai, China). Copper (II) nitrate trihydrate ($Cu(NO_3)_2{\cdot}3H_2O$, 99%), polyvinylpyrrolidone (PVP, average mol wt 40,000), 30% hydrogen peroxide (H_2O_2), $_L$-Cysteine (Cys), $_L$-Glutamine (Glu), glycine (Gly), $_L$-phenylalanine (Phe), Tyrosine (Tyr), adenosine 5′-triphosphate disodium salt hydrate (ATP) and $_{DL}$-Lactic were obtained from Shanghai Aladdin Biochemical Technology Co., Ltd. Alanine (Ala), $_L$-Histidine (His), $_L$-Lysine (Lys), dihydroxyphenylacetic acid (DOPAC), 5-hydroxytryptamine (5-HT), N, N-dimethylformamide (DMF) and ethanol were got from Meryer Chemical Technology Co., Ltd (Shanghai, China). CNTs were obtained from XFNANO Co., Ltd, China. All metal ion solutions are prepared from their chloride or nitrate salts from Meryer (Shanghai) Chemical Technology Co., Ltd.

In the selectivity experiments, ClO^- was derived from NaClO. $^1O_2$ was formed by the reaction of NaClO with H_2O_2. $^{\bullet}OH$ was generated by the reaction of H_2O_2 with Fe^{2+}. $ONOO^-$ was provided by $NaNO_2$ and H_2O_2. $ROO^{\bullet}$ was obtained by thermolysis of AAPH in air-saturated aqueous solution at 310 K. $O_2^{\bullet-}$ was derived from dissolved KO_2 in DMSO solution.

Synthesis of 2D Cu-TCPP(Fe) and 2D Cu-TCPP nanosheets. 2D Cu-TCPP(Fe) nanosheets were synthesized following the previous literature and some details were altered.[1] In brief, $Cu(NO_3)_2{\cdot}3H_2O$ (2.4 mg), trifluoroacetic acid (40 μL, 1.0 M) and PVP (10 mg) were dissolved in 12mL mixed solution of DMF (9 mL) and ethanol (3 mL). Afterwards, TCPP(Fe) (4.4 mg) dissolved in a mixture of DMF (3 mL) and ethanol (1 mL) was gradually introduced to the above solution by dropwise addition under stirring. Subsequently, the mixed solution was transferred into a 25 mL Teflon-lined stainless steel and sonicated for 15 min, followed by heating at 80 °C for 4 h. The resulting nanomaterial was washed twice with ethanol and centrifuged at 8000 r.p.m for 20 min. Finally, 2D Cu-TCPP(Fe) nanosheets were dispersed in ethanol for later use.

2D Cu-TCPP nanosheets were synthesized as previously reported.[2] In brief,

$Cu(NO_3)_2 \cdot 3H_2O$ (3.6 mg), trifluoroacetic acid (10 μL, 1.0 M) and PVP (10 mg) were dissolved in 12mL mixed solution of DMF (9 mL) and ethanol (3 mL). Then TCPP (4.0 mg) dissolved in a mixture of DMF (3 mL) and ethanol (1 mL) was gradually introduced to the above solution by dropwise addition under stirring. After that, the mixed solution was transferred into a 25 mL Teflon-lined stainless steel and sonicated for 15 min, followed by heating at 80 °C for 3 h. The resulting nanomaterial was washed twice with ethanol and centrifuged at 8000 r.p.m for 10 min. Finally, 2D Cu-TCPP nanosheets were dispersed in ethanol.

Synthesis of CNT/2D Cu-TCPP(Fe) and CNT/2D Cu-TCPP composites. Briefly, 2 mg CNT was dissolved in 1 mL DMF solution and sonicated for 15 min. 2D Cu-TCPP(Fe) (V_1) and CNT (V_2) dispersion were mixed at different volume ratios (3:1, 2:1, 1:1, 1:2), sonicated for 30 min and stirred for 12 h to obtain a series of uniform CNT/2D Cu-TCPP(Fe) dispersion for the optimization of experimental conditions. The synthesis of CNT/2D Cu-TCPP was the same as CNT/2D Cu-TCPP(Fe) except for changing 2D Cu-TCPP(Fe) into 2D Cu-TCPP.

Preparation and Modification of Electrodes. Carbon fiber microelectrode (CFME) was fabricated as follows. First of all, silver conductive adhesive was applied to the carbon fiber for the adhesion of a copper wire. Once dried, the carbon fiber along with the copper wire was cautiously transferred into a capillary. Finally, the capillary was securely sealed using epoxy resin and hardened in an oven. Under the observation of a microscope, the protruding carbon fiber was carefully trimmed to a length of 5 mm. Before undergoing any modifications, the CFMEs were sequentially sonicated in acetone, 3.0 M HNO_3, 1.0 M KOH, and distilled water each for 3 min to effectively remove impurities on the electrode surface. One drop of CNT/2D Cu-TCPP(Fe) dispersion prepared above was dripped onto a smooth glassy plate. The modification of CFME with CNT/2D Cu-TCPP(Fe) was achieved by meticulously immersing and rolling the electrodes into the droplet for approximately 1 min to obtain CFME/CNT/2D Cu-TCPP(Fe) modified electrode.

Instruments and Measurements. Transmission electron microscopy (TEM) was used to observe the morphology of 2D Cu-TCPP(Fe) and CNT/2D Cu-TCPP(Fe) composite on Tecnai G2 F20 (FEI, Netherlands). SEM was conducted with Nova Nano SEM 230.

Electrochemical experiments were carried out on an electrochemical analyzer (CHI832D, Chenhua, China). CFME, platinum (Pt) wire and Ag/AgCl electrode acted as working electrode, counter electrode and reference electrode, respectively. All experiments were performed at room temperature. The electrochemical parameters for differential pulse voltammetry (DPV) were chosen as follows: potential step of 0.004 V, modulation amplitude of 50 mV, pulse width of 0.06 s, sampling width of 0.02, pulse period of 1.0. The optimal electrochemical experimental conditions were investigated by changing the ratios of 2D Cu-TCPP(Fe) (V_1) and CNT (V_2), pH values and Cl^- concentrations. Quantitative analysis of GSH was realized by recording DPV curves under the optimal conditions.

In vivo experiments. All animal-related procedures were carried out with the approval of the Animal Ethics Committee at Tianjin Normal University, China. Male Wistar rats (300-400 g, Shanghai SLAC Laboratory Animal Co. Ltd., China) were employed in our experiments. The surgery was conducted as described previously.[3] In brief, rats were administered with chloral hydrate with an initial dose of 300 mg kg^{-1} (i.p.) and additional doses of 100 mg kg^{-1} (i.p.) as required to sustain anesthesia. The rats were positioned in a stereotaxic frame (Beijing Tide-Gene Biotechnology Development Center) with the incisor bar adjusted to 5 mm above the interaural line. Subsequently, holes were accurately drilled through the skull at the required locations. The CFME/CNT/2D Cu-TCPP(Fe) microelectrode was implanted in different regions in rat brain of the cortex (AP = 4.0 mm, L=3.0 mm from bregma, V=1.8 mm from the surface of skull, the right striatum (AP = 0.72 mm, L= 2.5 mm anterior to bregma, and V=5.0 mm from the surface of skull) and the dorsal hippocampus (AP = 4.0 mm, L = 3.0 mm from bregma, V = 2.9 mm from the surface of the skull). The reference and counter electrodes were inserted into a 2 mm plastic cannula, positioned at a distance of ~ 5 mm from the working electrode. Cerebral ischemia was conducted for in vivo analysis. The procedure of global cerebral ischemia is presented as follows. In brief, a midline cervical incision was performed for isolating the bilateral common carotid arteries. Both common carotid arteries were blocked using stainless steel vascular clips to induce ischemia. The clips were subsequently removed for reperfusion. CFME/CNT/2D Cu-TCPP(Fe) microelectrode should be replaced by a new one in advance for GSH monitoring under different ischemia times.

The microdialysis probe (CMA/110/111 Tub) was implanted in the three brain regions above to obtain microdialysis. To minimize the injury to the rat brain, the probe should be implanted carefully and slowly within 15 min. Throughout the surgery, the rat's body temperature was kept at 37°C using a homeothermic blanket. The probes were perfused with aCSF solution at 2 μL min^{-1} for at least 90 min for equilibration. Brain dialysates were collected under normal and ischemia conditions for HPLC analysis.

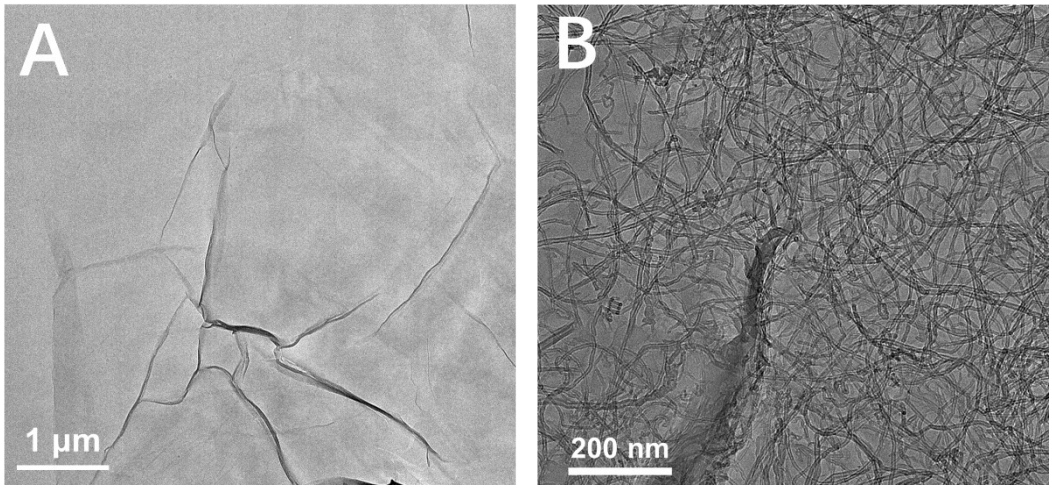

Figure S1. TEM images of (A) 2D Cu-TCPP(Fe) nanosheets and (B) CNT/2D Cu-TCPP(Fe) composites.

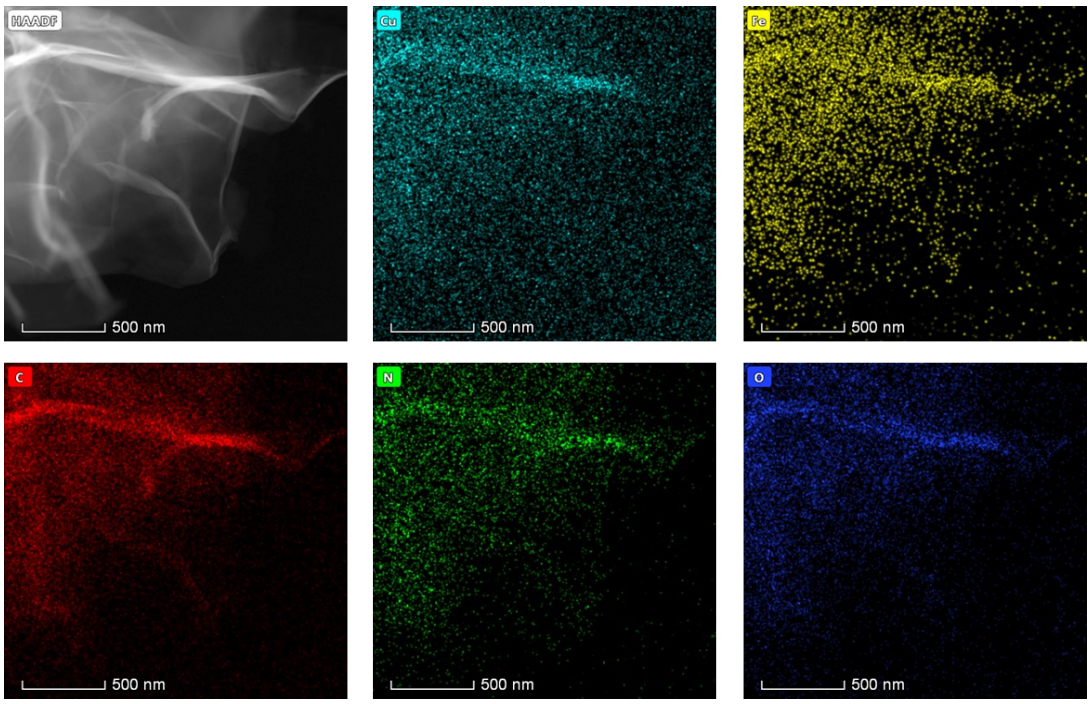

Figure S2. Elemental mapping of 2D Cu-TCPP(Fe) nanosheets.

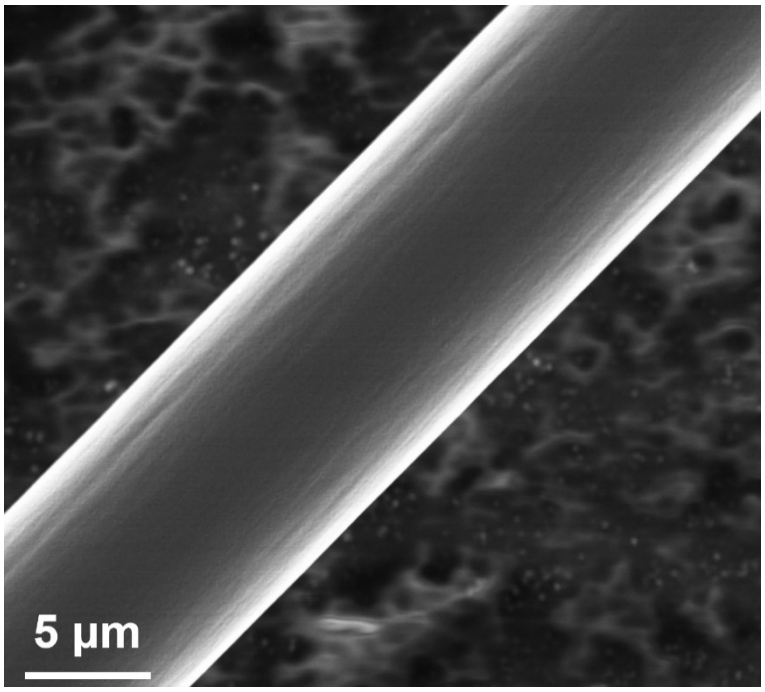

Figure S3. SEM image of bare CFME.

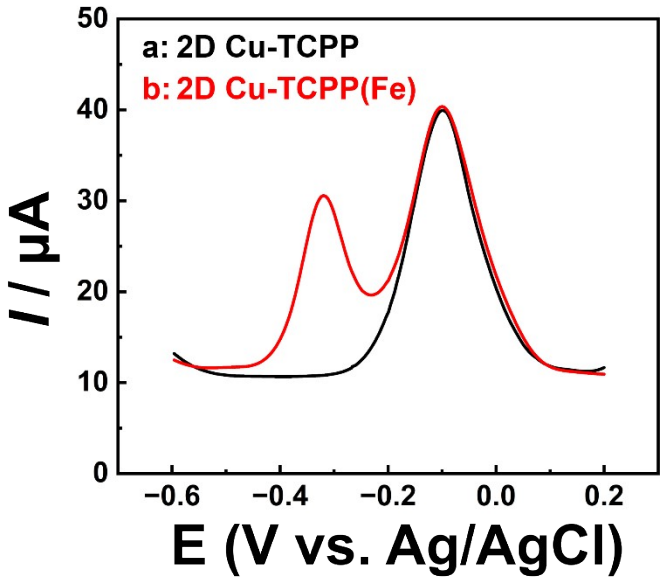

Figure S4. DPV curves obtained at CFME/CNT/2D Cu-TCPP (a) and CFME/CNT/2D Cu-TCPP(Fe) (b) modified electrodes in PBS solution (pH=7.4) containing 120 mM Cl^-.

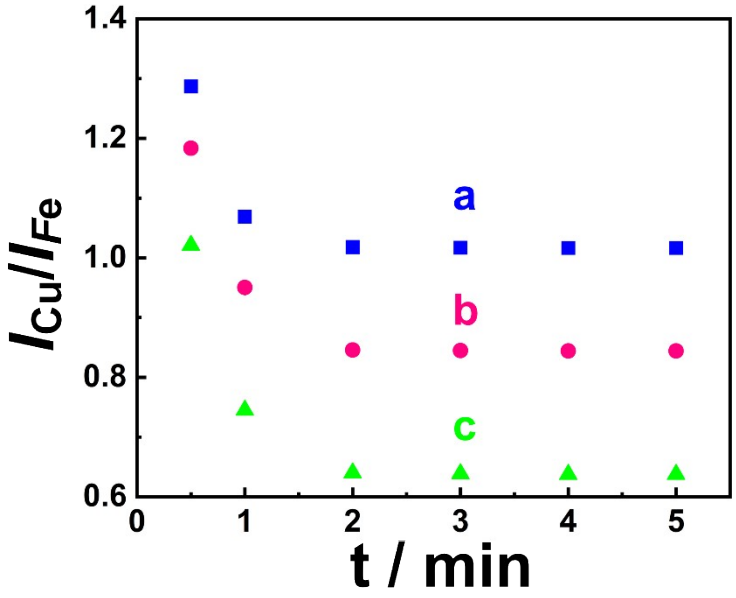

Figure S5. The relationship between current ratio of I_{Cu}/I_{Fe} and reaction time recorded at CFME/CNT/2D Cu-TCPP(Fe) in 0.1 M PBS (pH=7.4) containing 120 mM Cl^- upon the addition of varied concentrations of GSH (a. 1 nM, b. 100 nM and c. 10 μM).

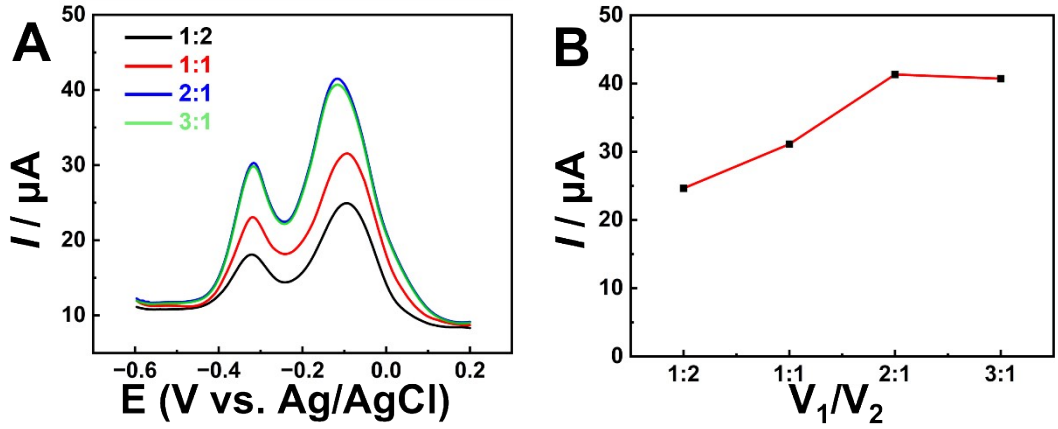

Figure S6. Effect of volume ratio of 2D Cu-TCPP(Fe) (V_1) and CNT (V_2) on the oxidation peak current of CuCl in 0.1 M PBS (pH=7.4) containing 120 mM Cl^-.

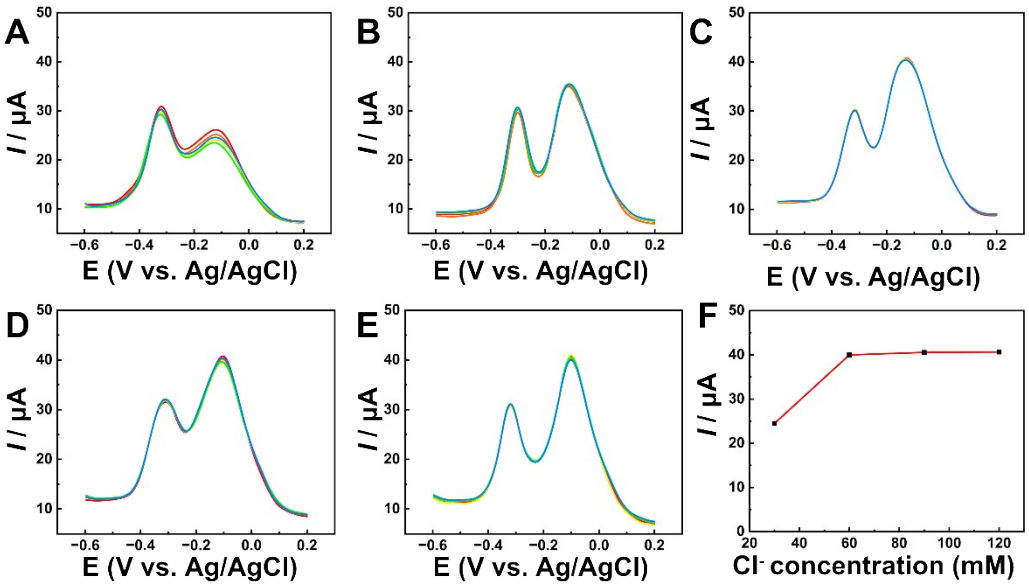

Figure S7. (A-E) Stability of CFME/CNT/2D Cu-TCPP(Fe) modified electrodes in PBS buffer (pH=7.4) containing different Cl^- concentrations: 0, 30 mM, 60 mM, 90 mM and 120 mM, respectively. (F) The effect of Cl^- concentration on oxidation peak current of CuCl.

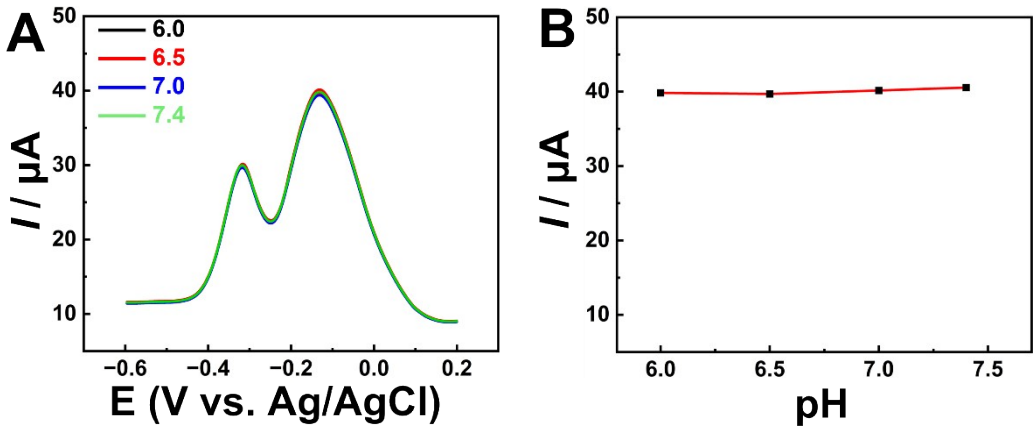

Figure S8. Effect of pH value on the oxidation peak current of CuCl in 0.1 M PBS (pH=7.4) containing 120 mM Cl^-.

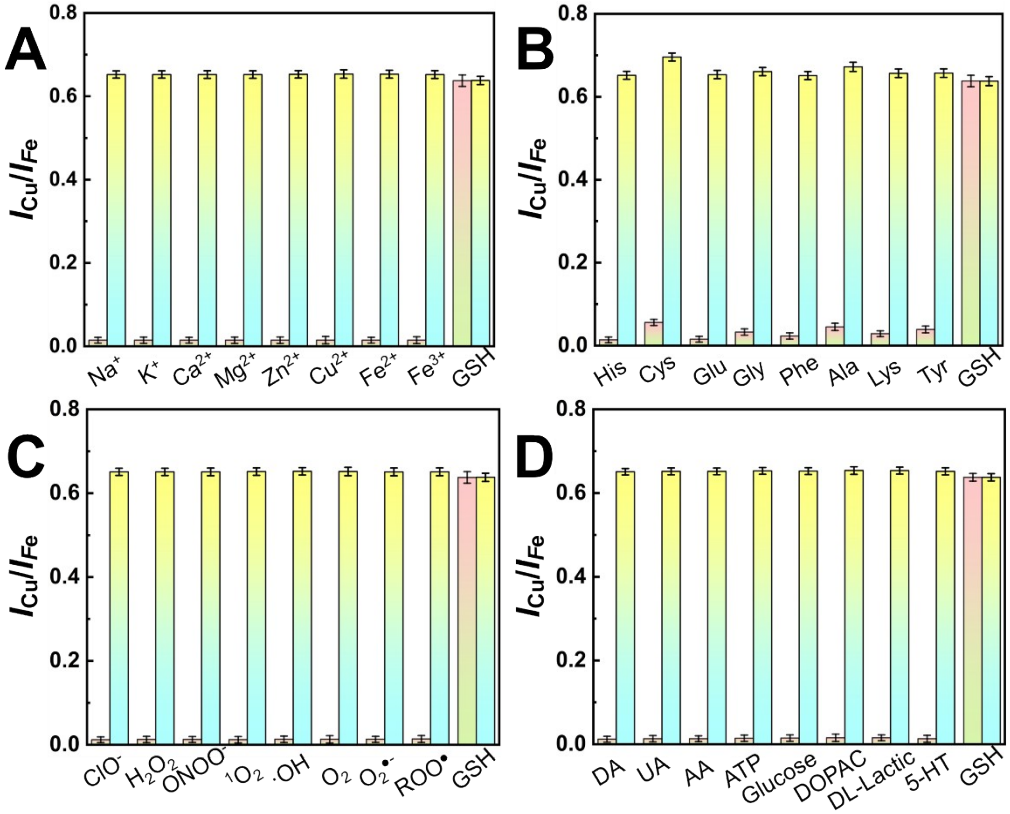

Figure S9. Selectivity and competition tests of (A) mental ions (150 mM for Na^+, 100 mM for K^+, 1 mM for Ca^{2+}, 1 mM for Mg^{2+} and 10 μM for Zn^{2+}, Cu^{2+}, Fe^{2+} and Fe^{3+}), (B) amino acids (10 μM for His, Cys, Glu, Gly, Phe, Ala, Lys and Tyr), (C) ROS (100 μM for O_2, 20 μM for ClO^-, H_2O_2, $ONOO^-$, $^{\bullet}OH$, $^1O_2$ and $O_2^{\bullet-}$) and (D) biological species (10 μM for UA and ATP, 1 μM for DA and DOPAC, 100 μM for AA, 1 mM for Glucose and $_{DL}$-Lactic and 5 μM for 5-HT) against 10 μM GSH.

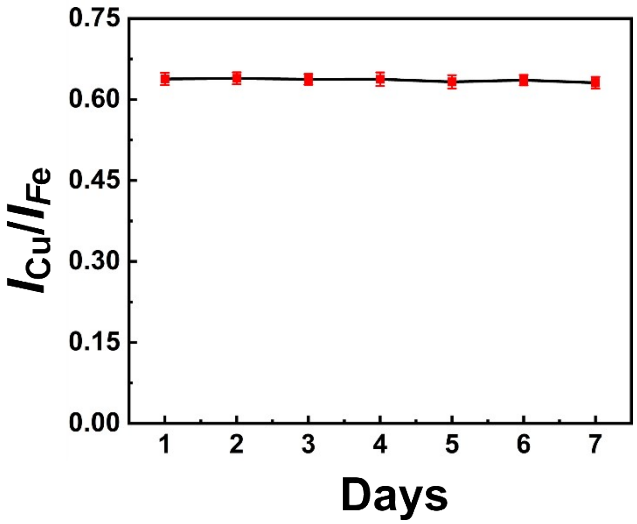

Figure S10. Stability tests for CFME/CNT/2D Cu-TCPP(Fe) modified electrodes after reacting with 10 μM GSH.

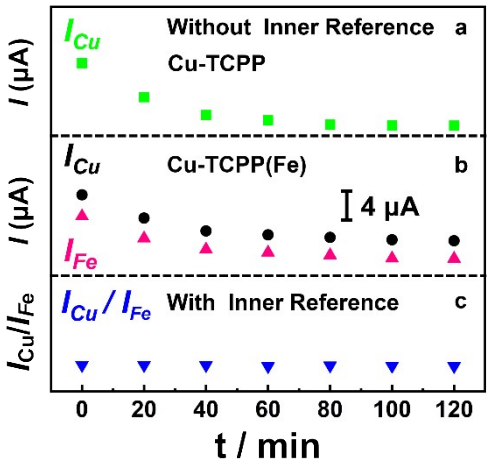

Figure S11. Comparison of the performance of sensors with and without the inner reference in PBS solution (pH=7.4, 120 mM Cl^-) containing rat brain homogenate: (a) peak current value of I_{Cu} at CFME/CNT/2D Cu-TCPP, (b) peak current values of I_{Cu} and I_{Fe} at CFME/CNT/2D Cu-TCPP(Fe), and (c) current ratio of I_{Cu} / I_{Fe} obtained at CFME/CNT/2D Cu-TCPP(Fe) electrode upon different times.

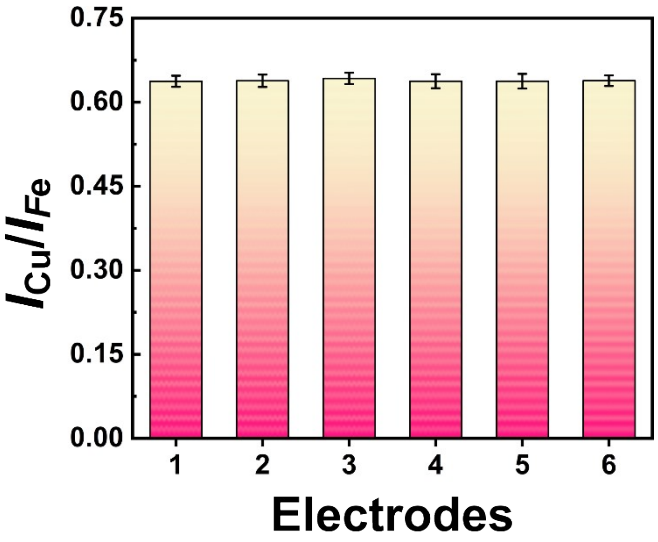

Figure S12. Reproducibility tests for CFME/CNT/2D Cu-TCPP(Fe) modified electrodes upon the addition of 10 μM GSH.

Table S1. Concentrations of GSH determined in striatum, hippocampus and cortex in live rat brains at different ischemia times.

Ischemia time	Region	GSH (μM)
		Rats (n=3)
0 min	Hippocampus	6.00±0.33
	Striatum	5.76±0.36
	Cortex	5.45±0.24
10 min	Hippocampus	4.30±0.31
	Striatum	4.02±0.43
	Cortex	2.98±0.31
20 min	Hippocampus	2.98±0.29
	Striatum	2.78±0.32
	Cortex	1.02±0.33
30 min	Hippocampus	1.48±0.32
	Striatum	1.22±0.35
	Cortex	0.95±0.34
40 min	Hippocampus	1.47±0.27
	Striatum	1.25±0.22
	Cortex	1.10±0.30

The *t* test and the statistical comparison.

The accuracy of the ratiometric electrochemical microsensor was assessed by taking the traditional HPLC as a standard method. A *t* test was conducted to evaluate whether the means obtained by the electrochemical method and HPLC method showed statistical difference. The *t* value was calculated according to equation 1

$$t = \frac{|\bar{X}_1 + \bar{X}_2|}{s} \times \sqrt{\frac{n_1 n_2}{n_1 + n_2}} \quad (1)$$

in which

$$s = \sqrt{\frac{s_1^2(n_1 - 1) + s_2^2(n_2 - 1)}{(n_1 - 1) + (n_2 - 1)}} \quad (2)$$

The standard deviations obtained by the present method and HPLC were represented as s_1 and s_2, respectively. n_1 and n_2 are the number of samples analyzed using the electrochemical method and HPLC (in this case, n_1 and n_2 are both equal to 3). If the calculated t value exceeds the standard t value of 2.13 (a = 0.1), it indicates that the results acquired from the electrochemical method are not highly consistent with those obtained from HPLC method.

Table S2. GSH concentrations in live rat brains determined by the present ratiometric biosensor compared with those obtained by HPLC in dialysates.

Region	the present method				HPLC			
	Rat 1	Rat 2	Rat 3	Mean±SD（n=3）	Rat 1	Rat 2	Rat 3	Mean±SD（n=3）
	GSH (μM)				GSH (μM)			
Normal								
Hippocampus	6.14	6.02	5.78	5.98±0.15	6.12	5.99	5.63	5.91±0.21
Striatum	5.93	5.35	5.86	5.71±0.26	5.90	5.88	5.31	5.70±0.27
Cortex	5.12	5.52	5.69	5.44±0.24	5.41	5.15	5.74	5.43±0.24
Ischemia								
Hippocampus	1.56	1.67	1.13	1.45±0.23	1.49	1.75	1.16	1.46±0.24
Striatum	1.56	1.11	0.97	1.21±0.25	1.54	0.81	1.34	1.23±0.31
Cortex	0.72	1.41	0.93	1.02±0.29	1.16	0.61	1.22	1.00±0.27

Reference

(1) Y. Wang, M. Zhao, J. Ping, B. Chen, X. Cao, Y. Huang, C. Tan, Q. Ma, S. Wu, Y. Yu, Q. Lu, J. Chen, W. Zhao, Y. Ying and H. Zhang, *Adv. Mater.,* 2016, **28**, 4149-4155.

(2) Y. Zeng, M. Wang, Z. Sun, L. Sha, J. Yang and G. Li, *J. Mater. Chem. B.,* 2022, **10**, 450.

(3) S. Huang, L. Zhang, L. Dai, Y. Wang and Y. Tian, *Anal. Chem.,* 2021, **93**, 5570−5576.