Supporting Information

Real-time Monitoring of Peroxynitrite ($ONOO^-$) in the Rat Brain by Developing a Ratiometric Electrochemical Biosensor

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1. Developed Ratiometric Electrochemical for Real-Time Monitoring of $ONOO^-$

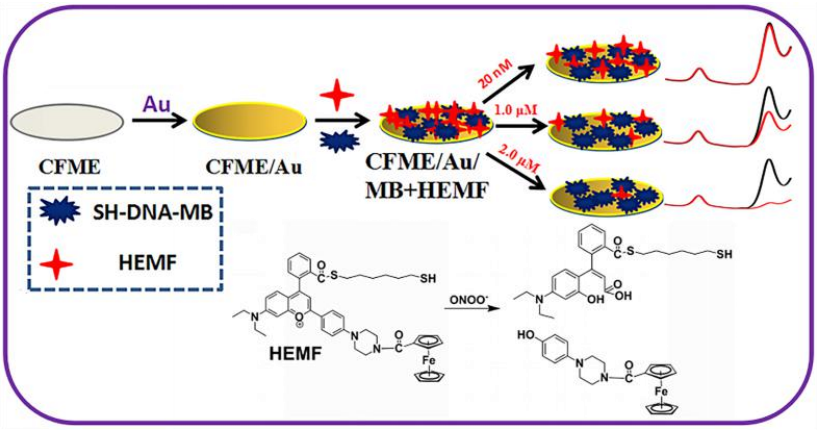


Figure S1. Developed Ratiometric Electrochemical for Real-Time Monitoring of $ONOO^-$.

2. Synthetic route of HEMF

Synthesis of HEMF. 2-(4-Diethylamino-2-hydroxybenzoyl) benzoic acid (313.0 mg, 1.0 mmol) and 4'-piperazinoacetophenone (204.0 mg, 1.0 mmol) in methanesulfonic acid (8.0 mL) were stirred at 100 °C for 4 h at Ar atmosphere. After 4 h, the reaction product was cooled to room temperature. Then the mixture was extracted with dichloromethane (CH_2Cl_2) three times. The organic layers were collected, and then dried over Na_2SO_4. Finally, the mixture was evaporated under reduced pressure, and then purified by preparative TLC (CH_2Cl_2/EtOH=30:1) to offer compound 2 as a red solid (412.9 mg, 85.6%). A mixture of ferrocenecarboxylic (115.0 mg, 0.5 mmol), HATU (190.1 mg, 0.5 mmol) and triethylamine (20 μL) in 10.0 mL anhydrous CH_2Cl_2 was stirred for 2 h, then compound 2 (241.2 mg, 0.5 mmol) was added into the mixture under Ar atmosphere. The mixture was stirred at room temperature for 12 h. Then, the solvent was removed under vacuum condition, and the mixture was purified by preparative TLC (CH_2Cl_2/EtOH=40:1) to offer compound 3 as a blue solid (194.4 mg, 56.0%). A mixture of the corresponding compound 3 (173.6 mg, 0.25 mmol), 1,6-hexanedithiol (37.6 mg, 0.25 mmol), EDC (47.9 mg, 0.25 mmol), DMAP (12.2 mg,0.1 mmol) was added CH_2Cl_2 (20.0 ml) at room temperature. The mixture was stirred for 12 h. Then solvent was removed under reduced pressure and the mixture was purified by preparative TLC (CH_2Cl_2/EtOH=40:1, CH_2Cl_2/EA/EtOH=6:3:1) to offer HEMF as a blue solid (141.3 mg, 68.4%).

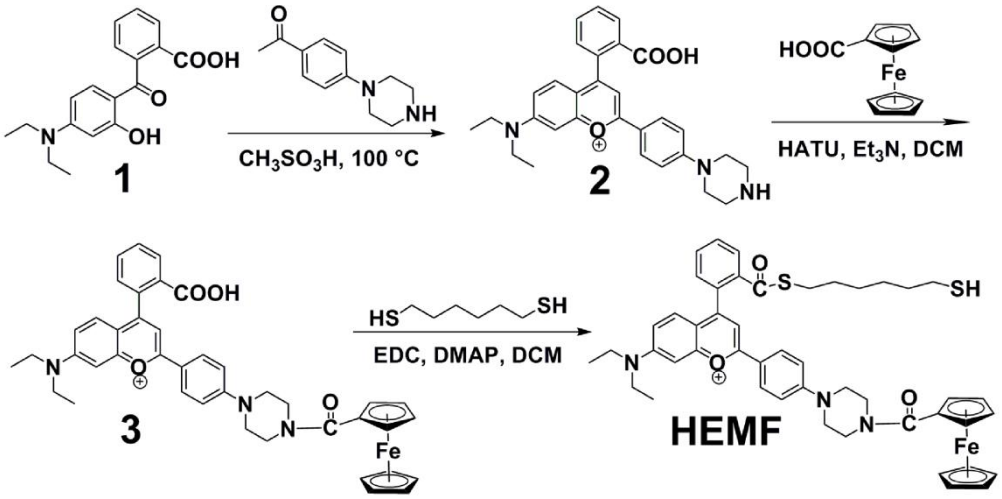
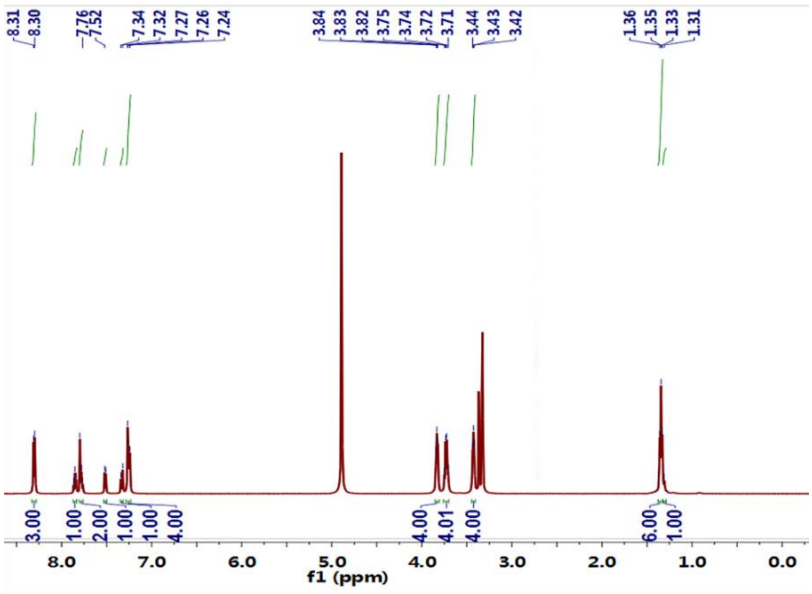
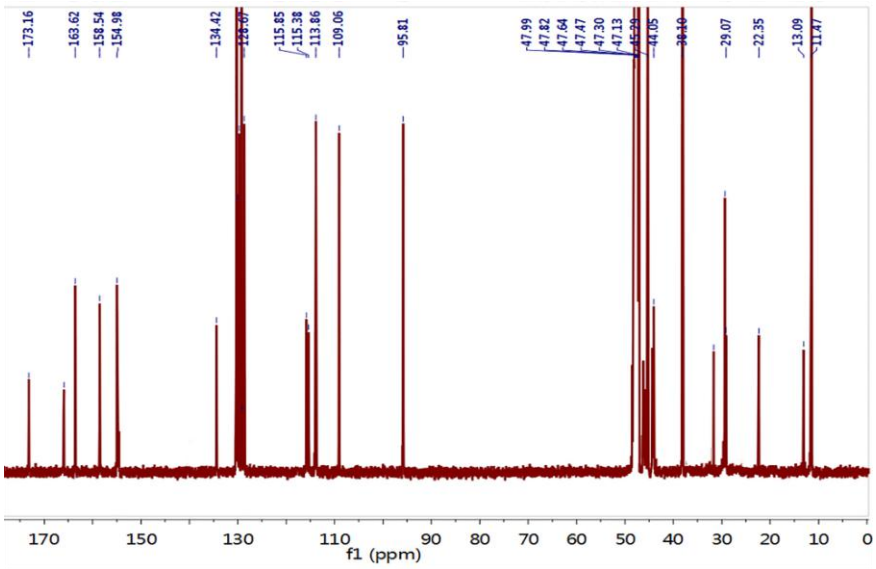


Figure S2. Synthetic route of HEMF.

3. ^{1}H NMR and ^{13}C NMR of Compound 2



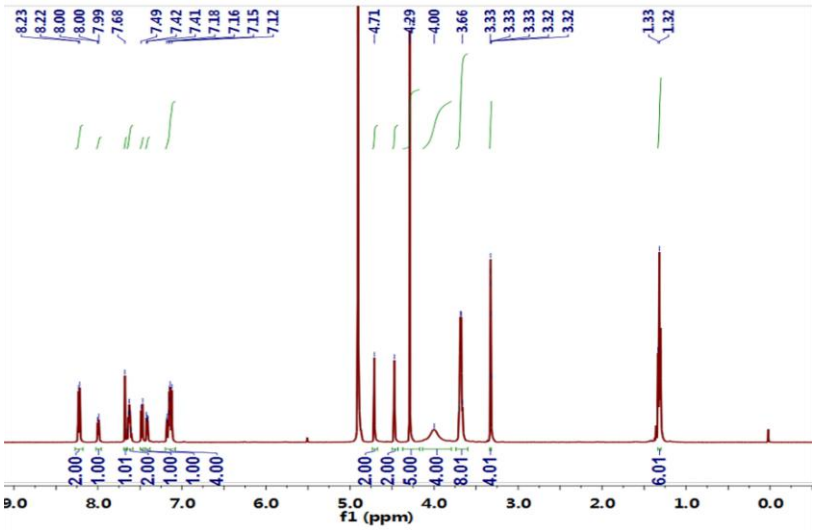
^{1}H NMR (500 MHz, CD_3OD) δ 8.30 (d, 3H), 7.85 (t, 1H), 7.80 – 7.76 (m, 2H), 7.51 (d, 1H), 7.33 (d, 1H), 7.27 - 7.22 (m, 4H), 3.83 (t, 4H), 3.73 (m, 4H), 3.43 (t, 4H), 1.35 (t, 6H), 1.30 (s, 1H).



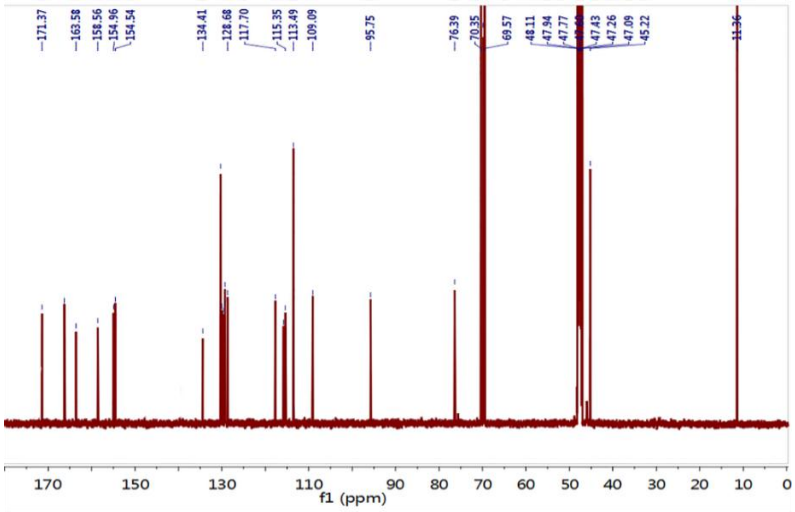
^{13}C NMR (126 MHz, CD_3OD) δ 173.1, 165.9, 163.6, 158.5, 154.9, 134.4, 130.2, 130.0, 129.7, 129.1, 128.6, 115.8, 115.3, 113.8, 109.0, 95.8, 45.2, 44.0, 38.1, 31.6, 29.3, 29.0, 22.3, 13.0, 11.4.

Figure S3. ^{1}H NMR and ^{13}C NMR of Compound 2.

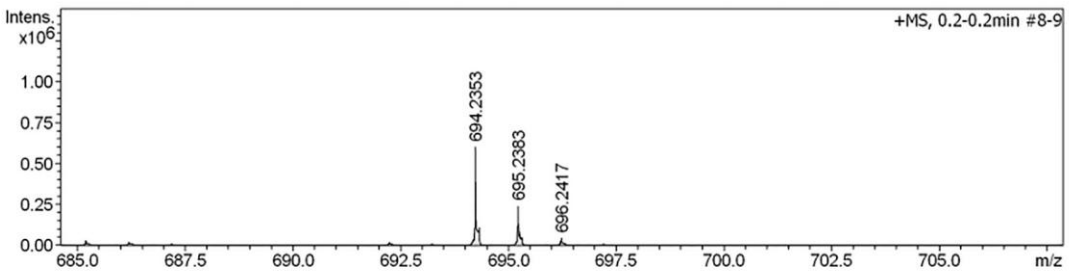
4. ^{1}H NMR, ^{13}C NMR and Mass spectrum of Compound 3



^{1}H NMR (500 MHz, CD_3OD) δ 8.22 (d, 1H), 8.00 (m, 1H), 7.68 (s, 1H), 7.65 - 7.60 (m, 2H), 7.48 (d, 1H), 7.42 - 7.40 (m, 1H), 7.18 - 7.12 (m, 4H), 4.71 (m, 2H), 4.47 (m, 2H), 4.29 (d, 5H), 4.00 (s, 4H), 3.70 - 3.66 (dd, 8H), 8.22 (d, 1H), 3.33 (m, 4H), 1.30 (t, 6H).



^{13}C NMR (126 MHz, CD_3OD) δ 171.3, 166.2, 163.5, 158.5, 154.9, 154.5, 134.4, 130.3, 129.7, 129.2, 128.6, 117.7, 115.7, 115.3, 113.4, 109.0, 95.7, 76.3, 70.3, 69.8, 69.5, 45.2, 11.3.

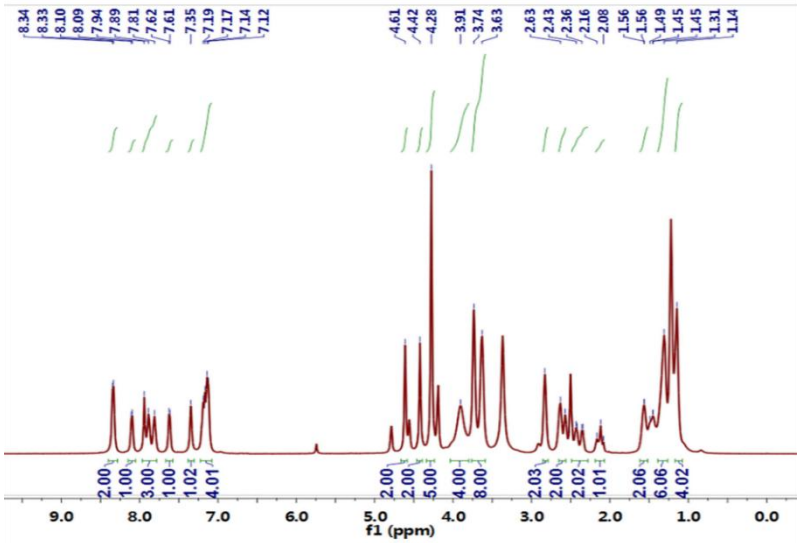


#	m/z	Res.	S/N	I	I %	FWHM
1	694.2353	45184	4923.9	601004	100.0	0.0154
2	695.2383	35474	2000.6	244196	40.6	0.0196
3	696.2417	25725	373.3	45554	7.6	0.0271

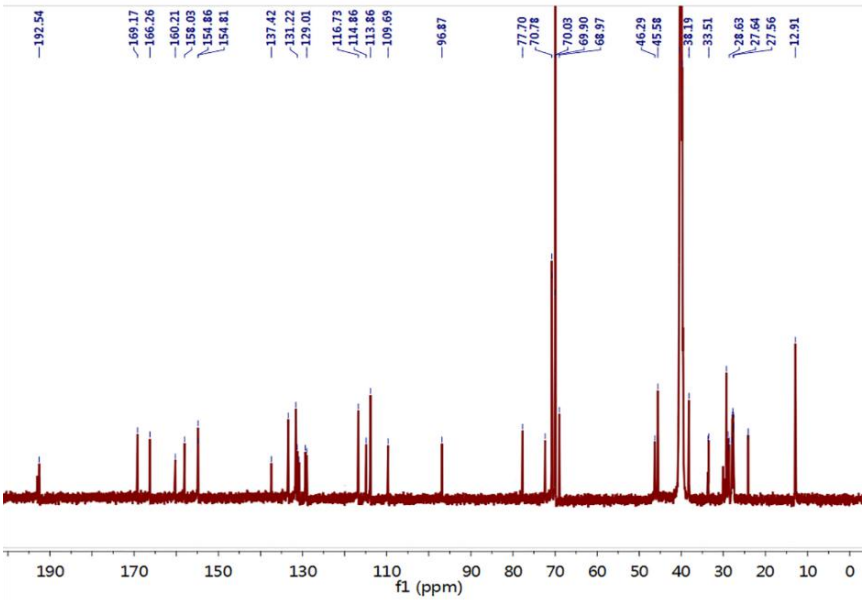
ESI-MS $C_{41}H_{40}FeN_3O_4^+$ [M^+], found 694.2353.

Figure S4. ^{1}H NMR, ^{13}C NMR and Mass spectrum of Compound 3.

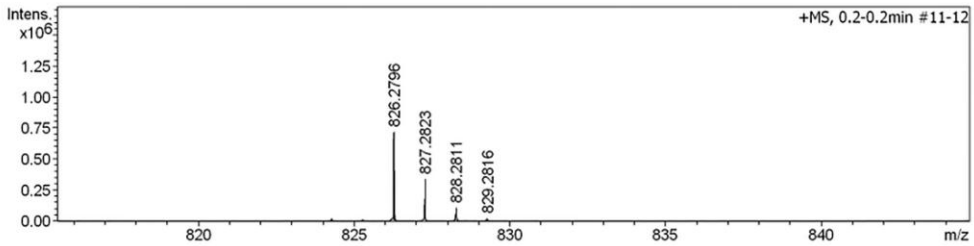
5. ^{1}H NMR, ^{13}C NMR, mass spectra and FTIR spectrum of HEMF



^{1}H NMR (500 MHz, DMSO) δ 8.33 (d, 2H), 8.09 (d, 1H), 7.94 - 7.81(m, 3H), 7.61 (d, 1H), 7.35 (s, 1H), 7.19 - 7.12 (m, 4H), 4.61 (d, 2H), 4.42 (s, 2H), 4.28 (s, 5H), 3.91 (s, 4H), 3.74 - 3.63 (d, 8H), 2.83 (s, 2H), 2.63 - 2.57 (m, 2H), 2.43 - 2.35 (m, 2H), 2.12 (m, 1H), 1.55 (s, 2H), 1.31 (t, 6H), 1.14 (s, 4H).

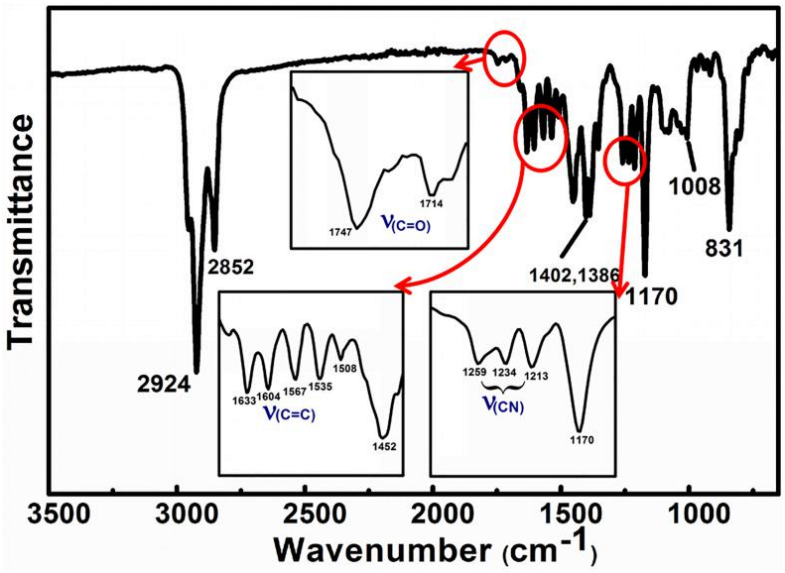


^{13}C NMR (126 MHz, DMSO) δ 192.5, 169.1, 166.2, 160.2, 158.0, 154.8, 154.8, 137.4, 131.2, 129.0, 116.7, 114.8, 113.8, 109.6, 96.8, 77.7, 72.3, 70.7, 70.6, 69.9, 68.9, 46.2, 45.5, 38.1, 33.6, 33.5, 29.3, 28.8, 28.3, 27.9, 27.8, 27.7, 27.6, 27.6, 27.5, 24.1, 24.1, 12.9.



#	m/z	Res.	S/N	I	I %	FWHM
1	826.2796	48003	8470.8	718312	100.0	0.0172
2	827.2823	39450	3996.4	338735	47.2	0.0210
3	828.2811	32232	1258.5	106633	14.8	0.0257
4	829.2816	26733	252.0	21337	3.0	0.0310

ESI-MS $C_{47}H_{52}FeN_3O_3S_2^+$ [M^+], found 826.2796.



FTIR spectrum of HEMF.

Figure S5. ^{1}H NMR, ^{13}C NMR, Mass spectrum and FTIR spectrum of HEMF.

6. Comparison of DPVs obtained at CFME/Au/MB+HEMF and Au/MB+HEMF electrode

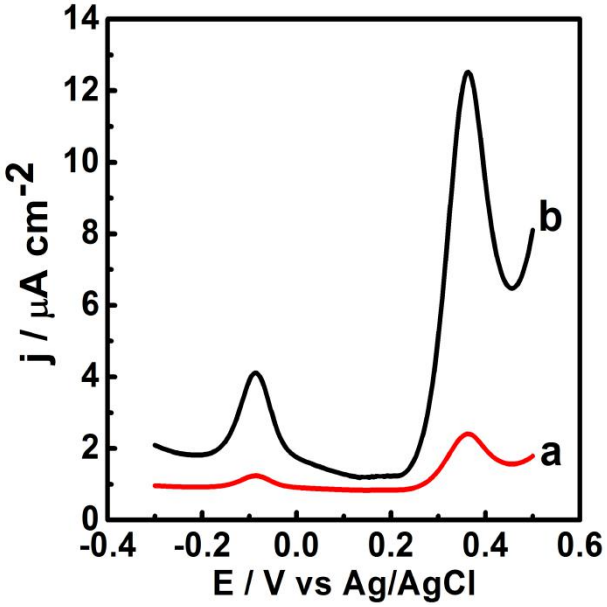


Figure S6. DPVs obtained at (a) CFME/Au/MB+HEMF, (b) Au/MB+HEMF electrode in 0.1 M PBS (pH 7.4) at scan rate of 100 mV s^{-1}.

7. XPS characterization of electrode modification processes

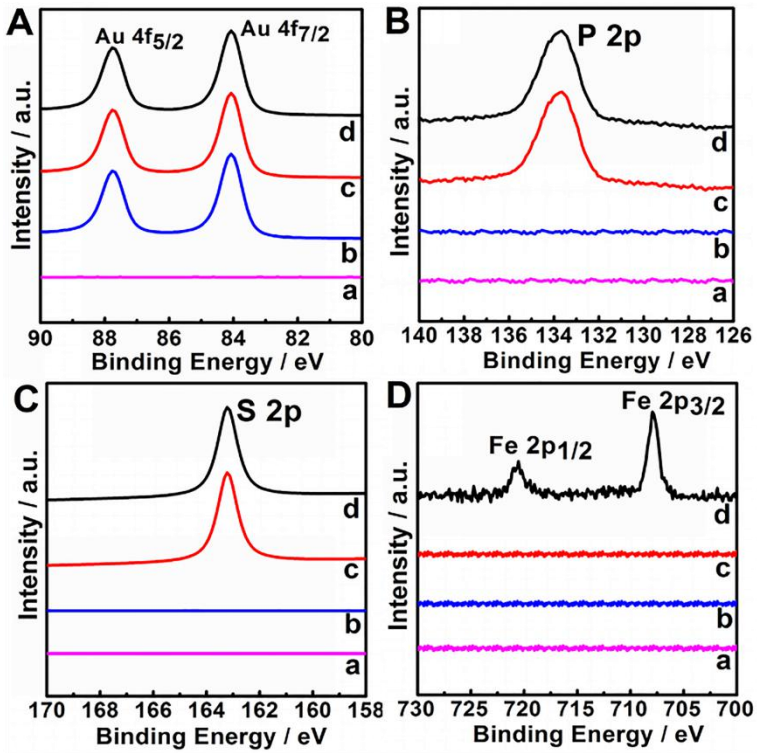


Figure S7. XPS spectra of (A) Au $4f_{7/2}$ and Au $4f_{5/2}$, (B) P 2p, (C) S 2p and (D) Fe $2p_{3/2}$ and Fe $2p_{1/2}$ for (a) CFME, (b) CFME/Au, (c) CFME/Au/MB and (d) CFME/Au/MB+HEMF electrode.

8. Liquid chromatography-mass spectrum of $ONOO^-$ reacted with HEMF

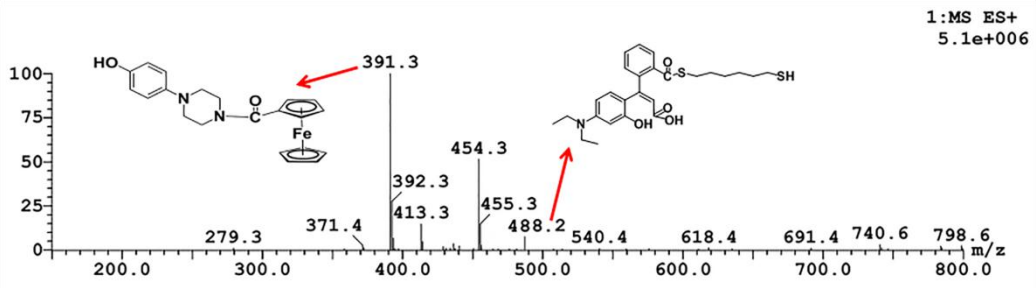


Figure S8. Liquid chromatography-mass spectrum of $ONOO^-$ reacted with HEMF.

9. The response time of CFME/Au/MB+HEMF electrode toward different concentrations of $ONOO^-$

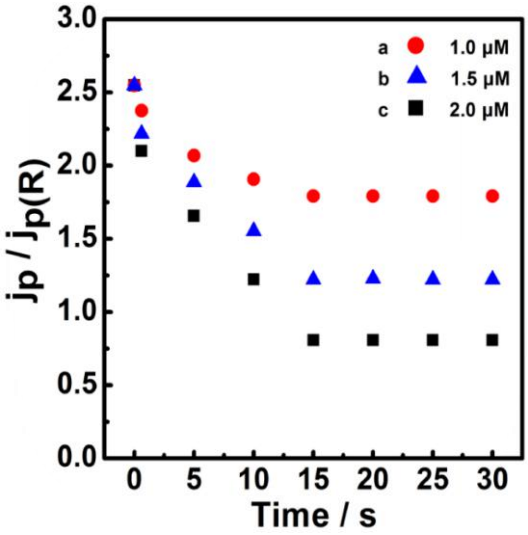


Figure S9. The relationship between $j_p/j_{p(R)}$ value and time obtained at CFME/Au/MB+HEMF electrode with addition of different concentrations of $ONOO^-$ (a: 1.0 μM; b: 1.5 μM, c: 2.0 μM) in 0.1 M PBS (pH 7.4).

10. DPVs obtained at CFME/Au/MB+HEMF electrode in aCSF (pH 7.4) with addition of different concentrations of $ONOO^-$

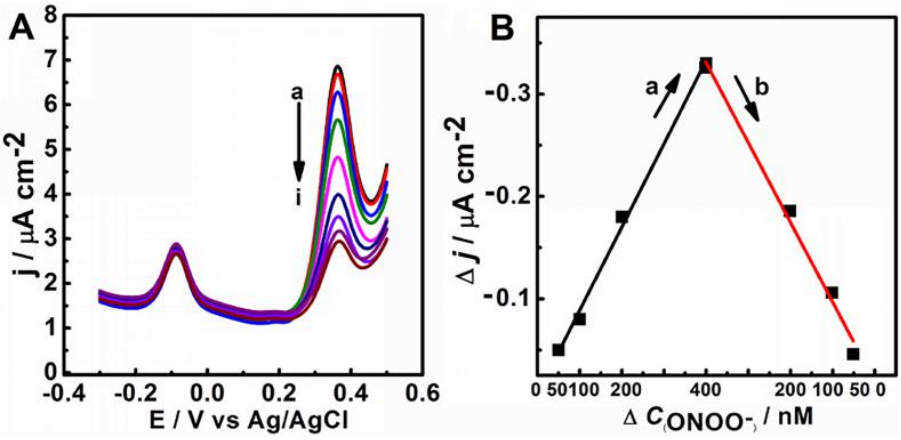


Figure S10. (A) DPVs obtained at CFME/Au/MB+HEMF electrode in aCSF (pH 7.4) with addition of different concentrations of $ONOO^-$ (From a to i: 0, 50, 100, 200, 400, 400, 200, 100, 50 nM). (B) The linear relationship of the peak current difference (Δj) obtained at 364 mV vs Ag/AgCl with different concentration differences of $ONOO^-$.

(Δj versus $\Delta C_{(ONOO^-)}$), (a, black) the concentration difference of $ONOO^-$ from low to high (50 nM to 400 nM), (b, red) the concentration difference of $ONOO^-$ from high to low (400 nM to 50 nM).

11. Stability of CFME/Au/MB+HEMF electrode (Figure S11-S13)

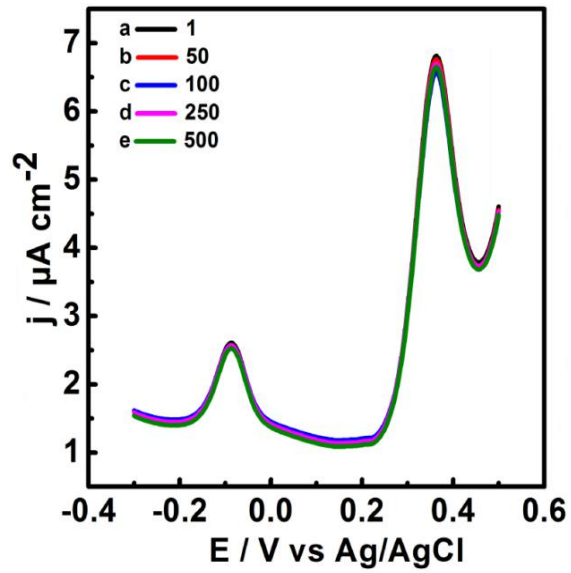


Figure S11. DPVs obtained at CFME/Au/MB+HEMF electrode in 0.1 M PBS (pH 7.4) by scanning after (a) the first cycle, (b) 50 cycles, (c) 100 cycles, (d) 250 cycles, and (e) 500 cycles, respectively.

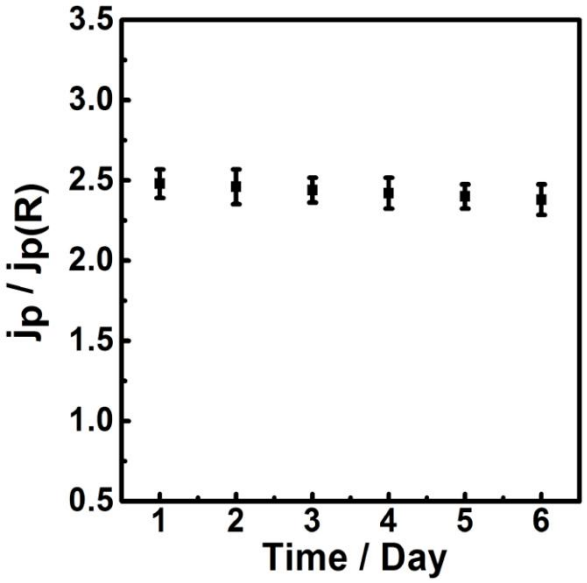


Figure S12. Stability test for CFME/Au/MB+HEMF electrode in 0.1 M PBS (pH 7.4) over 6 days.

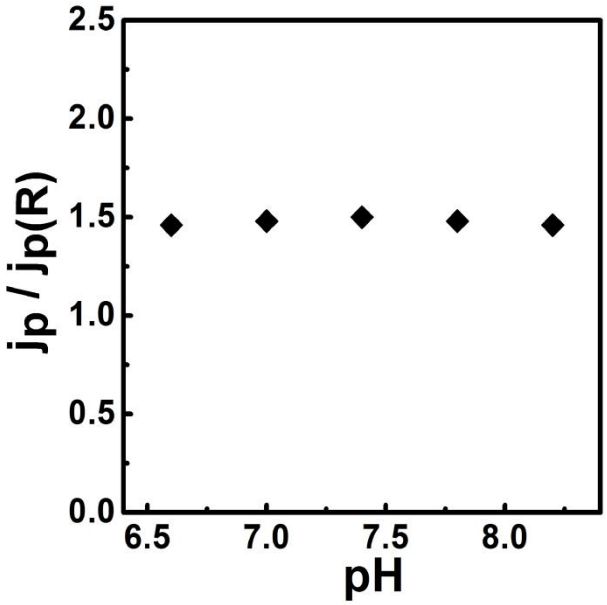


Figure S13. The $j_p/j_{p(R)}$ value of 1.0 μM $ONOO^-$ under different pH values (6.6, 7.0, 7.4, 7.8, 8.2).

12. Reproducibility test for CFME/Au/MB+HEMF electrodes

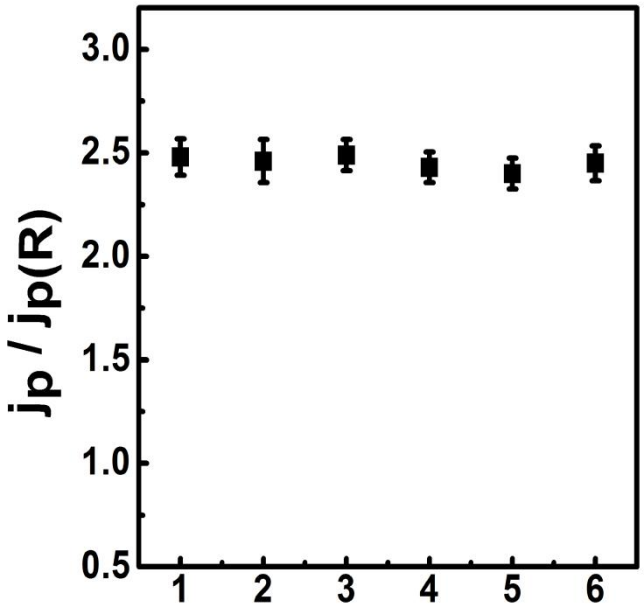


Figure S14. Reproducibility test for different CFME/Au/MB+HEMF electrodes in aCSF (pH 7.4).

13. MTT assay

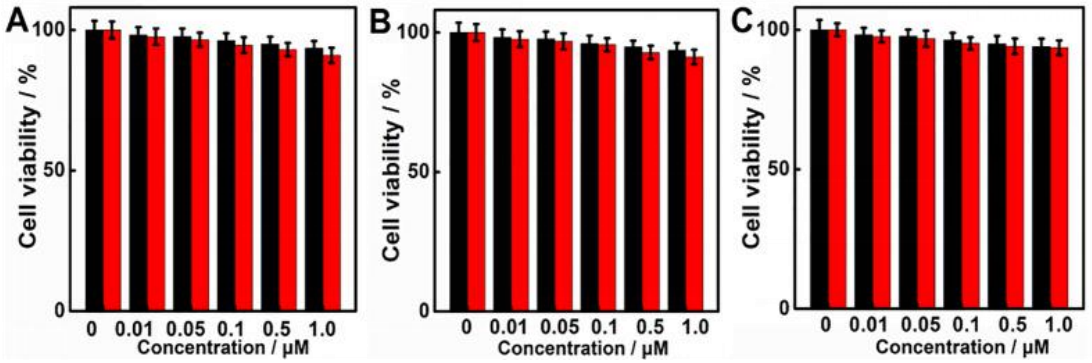


Figure S15. Viabilities of neurons after incubation for 24 h (black bars) and 48 h (red bars) with different concentrations of (A) HEMF, SH-DNA-MB (B) and (C) ferrocene which may fall into the rat brain after reacted with $ONOO^-$.

14. Apoptosis assay

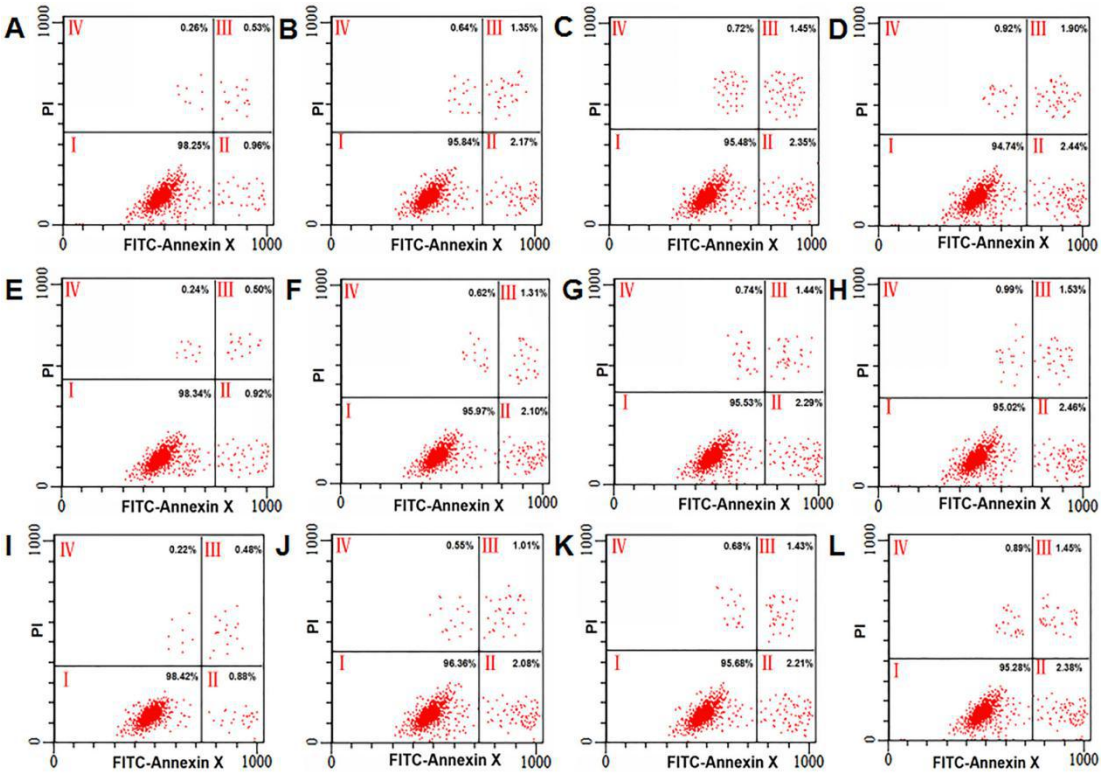


Figure S16. Apoptosis assay of neurons incubated with the HEMF (A, B, C, D), SH-DNA-MB (E, F, G, H) and ferrocene which may fall into the rat brain after reacted with $ONOO^-$(I, J, K, L) at concentrations of 0 μM (A, E, I), 0.01 μM (B, F, J), 0.1 μM (C, G, K) and 1.0 μM (D, H, L). I, II, III, and IV represents the region of normal cells, early apoptotic cells, late apoptotic cells, and dead cells, respectively.