Electronic Supplementary Information

**In Vivo Monitoring of Local pH Values in a Live Rat Brain Based on Design of Specific Electroactive Molecule for H\(^{+}\)**

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1. Experimental section

Reagents and Chemicals. 2,6-diaminopyridine and ferrocenecarboxylic acid were purchased from Tokyo Chemical Industry Co. Ltd. 3-mercaptopropionic acid (MPA), FcHT, 1-octadecanethiol (ODT), HAuCl₄·3H₂O, D(+) glucose, 3-MT, 5-HIAA, HVA, DL-lactic acid, ATP, tyramine and amino acids were purchased from Sigma Aldrich. Phosphate buffer solution (PBS) was made from KH₂PO₄, K₂HPO₄·3H₂O and KCl. HCl was used to adjust the pH of the solution. AA, DOPAC, KO₂, triethylamine, 2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) and other metal ions were purchased from Sinopharm Chemical Reagent Co. Ltd. NaClO, 30% H₂O₂, and 2, 2′-azobis(2-methylpropionamide)-dihydrochloride (AAPH) were purchased from Aladdin Chemistry Co. Ltd (China). UA were purchased from Alfa Aesar. All the reagents were analytical grade and used as received. In the selectivity test, O₂⁻ was generated from dissolved KO₂ (10 μM) in the DMSO solution. ROO⁻ was generated by thermolysis of AAPH (10 μM) in air-saturated aqueous solution at 310 K. ClO⁻ was derived from NaClO (10 μM). 'OH was provided by the reaction of H₂O₂ and Fe²⁺ (Fe²⁺: H₂O₂ = 1: 6). ¹⁰₂ was generated by the reaction of NaClO (10 μM) and H₂O₂ (10 μM). ONOO⁻ was provided by the reaction of NaNO₂ (10 μM) and H₂O₂ (10 μM).

Synthesis of Fe-Py. According to the procedures (Fig. S1, ESI†), ferrocenecarboxylic acid (0.460 g, 2 mmol), triethylamine (556 μL, 4 mmol) and HATU (2.2 mmol, 0.836 g) dissolved in 10 mL DMF were added in a small flask under stirring. The flask was kept at 50°C for 1 h. Then 2, 6-diaminopyridine (0.218 g, 2 mmol) was added in the mixture solution, the flask was kept at 50°C for 12 h. Then, the solution was extracted by ethyl acetate and the solvent was removed by rotary evaporation. Finally, the solution was purified by silica gel (100-200 meshes) column chromatography using
ethyl acetate-petroleum ether (5:1) as eluent. The compound was characterized by NMR and mass spectrometry (MS). $^1$H NMR (300 MHz, DMSO-d$_8$): δ(ppm) = 9.35 (s, 1H), 7.38 (d, 1H), 7.28 (d, 2H), 6.2 (d, 2H), 5.75 (s, 2H), 5.07 (s, 2H), 4.43 (s, 2H), 4.20 (m, 4H) (Fig. S2, ESI†); $^{13}$C NMR (500 MHz, CDCl$_3$): δ(ppm) = 168.8, 157.1, 150.0, 140.2, 104.0, 103.3, 75.7, 71.2, 67.0, 68.4 (Fig. S3, ESI†). TOF MS EI+: calculated for [M + H]$^+$ 321, found 321 (Fig. S4, ESI†).

**Preparation and Modification of Electrodes.** Carbon fiber electrode (CFME) was prepared as follows. Firstly, carbon fiber was connected to a copper weir with silver conductive adhesive. After the adhesive dried, the carbon fiber attached with copper wire was carefully moved into a capillary. Finally, the capillary was sealed with epoxy resin and solidified in oven. The protrusive carbon fiber was cut to a length of 5 mm under a microscope. Prior to modification, CFMEs were sonicated in acetone, 3.0 M HNO$_3$, 1.0 M KOH, and distilled water sequentially each for 3 min to remove the impurities on the surface of electrode. Then, by applying a potential of -0.2 V vs Ag/AgCl for 120 s, bamboo shoot-like gold nanostructures were electrochemically deposited on CFMEs from 0.1 M HClO$_4$ solution. The electrodeposited electrode was referred to CFME/Au. On the one hand, MPA was assembled on the electrode surface by immersing the CFME/Au electrode in an aqueous solution containing 10 mM MPA, then the electrode was rinsed with Mili-Q water and then transferred into ethanol solution containing 10 mM Fc-Py for 24 h. The MPA-modified electrode was denoted as CFME/Au/MPA. By using EDC and NHS as catalysts, Fc-Py was immobilized on the electrode through the interaction between –COOH group of MPA and –NH$_2$ group of Fc-Py. And the electrode was defined as CFME/Au/MPA/Fc-Py. On the other hand, for preparation the inner reference electrode, CFME/Au electrode was immersed in ethanol solution containing optimized concentration ratio of FcHT and ODT for 30 min, then rinsed with ethanol. The modified electrode was referred to
CFME/Au/FcHT. The cyclic voltammograms responses of CFME/Au electrode were obtained in 0.5 M H₂SO₄ solution to calculate the real electrode surface by integrating the cathodic peak area.

**Instruments and Measurements.** A two-channel electrochemical workstation (CHI 1040C, Shanghai, China) was used for the electrochemical experiments. CFME/Au/MPA/Fc-Py electrode served as the working electrode (channel 1), while CFME/Au/FcHT electrode was used as an inner reference electrode (channel 2). A KCl-saturated Ag/AgCl electrode and a platinum wire were employed as reference electrode and counter electrode respectively. Differential pulse voltammetry (DPV) was carried out in 10 mM PBS. Scanning electron microscopy (SEM) (S-4800, Hitachi, Japan) was used for direct observation of gold nanostructures deposited on CFME. X-ray photoelectron spectroscopy (XPS, AXIS Ultra DLD, Japan) was used to characterize each modification process. X-ray diffraction (XRD) was carried out by a Shimadzu XRD-6000. NMR spectra was obtained on a Bruker AV-400 spectrometer.

**In Vivo Experiments.** All procedures involving animals were conducted with the approval of the Animal Ethics Committee in East China Normal University, China. Male Wistar rats (300-400 g, Shanghai SLAC Laboratory Animal Co. Ltd., China) were used in our experiments. The surgery was performed as previously reported. Briefly, rats were anesthetized with chloral hydrate (initial dose of 300 mg kg⁻¹ (i. p.) with additional doses of 100 mg kg⁻¹(i. p.)) as demanded to maintain anesthesia. The rats were placed in a stereotaxic frame (Beijing Tide-Gene Biotechnology Development Center) with the incisor bar set at 5 mm above the interaural line and appropriately placed holes were drilled through the skull. The CFME/Au/MPA/Fc-Py and CFME/Au/FcHT electrodes were implanted in different regions in rat brain. According to standard stereotaxic procedures, the right striatum (AP = 0 mm, L= 2.5 mm anterior to bregma, and V=7.0 mm from the surface of skull), the dorsal hippocampus (AP = 5.0 mm, L = 5.0 mm from bregma, V = 2.5 mm from the
surface of the skull), the cortex (AP = 0.2 mm, L=5.6 mm from bregma, V=3.0 mm from the surface of skull) were detected respectively. The reference and counter electrodes, introduced in a 2 mm plastic cannula, located far from the working electrode (~5 mm). During in vivo experiments, surgeries for the cerebral ischemia were performed. Throughout the surgery, the body temperature of the rats was maintained at 37°C using a heating pad and injection of chloral hydrate (100 mg/kg) was given as needed. The details about global cerebral ischemia are demonstrated as follows: The first isolation of the bilateral common carotid arteries was followed by midline cervical incision. Afterwards, it could be clearly seen that the atlanto-occipital membrane was located amid the left side by retraction of the trachea and esophagus on the right. The basilar artery was occluded by a vascular clip (0.2 mm diameter) stainless steel, with a tapered blade tip. When the pause of blood flow was visually verified in the basilar artery, the occlusion of both common carotid were achieved via two Yasargil miniclips, which were removed after 30 mins’ reperfusion. It spent about 15 minutes in surgical procedure to the final clip. Ischemic duration from the launch of the last clip to the left common carotid artery was measured.

2. Synthetic route for Fc-Py (Figure S1)

![Figure S1. Synthetic route for Fc-Py](image)
3. NMR and MS data of Fc-Py (Figure S2-S4)

Figure S2. $^1$H NMR spectrum (300 MHz) of Fc-Py in DMSO-d6.
Figure S3. $^{13}$C NMR spectrum (500 MHz) of Fc-Py in CDCl$_3$.

Figure S4. Elemental composition search report by HR-MS for Fc-Py.
4. X-ray spectroscopic (XPS) spectra for different modified electrodes (Figure S5)

Figure S5. XPS spectra of (A) Au 4f\(_{7/2}\) and Au 4f\(_{5/2}\), (B) S 2p, (C) N 1s, (D) Fe 2p\(_{3/2}\) and Fe 2p\(_{1/2}\) for (a) CFME, (b) CFME/Au, (c) CFME/Au/MPA and (d) CFME/Au/MPA/Fc-Py.
5. Differential pulse voltammograms (DPVs) obtained at different modified CFMEs (Figure S6)

![Graph showing DPVs](image)

*Figure S6.* DPVs obtained at (a) bare CFME, (b) CFME/Au, (c) CFME/Au/MPA and (d) CFME/Au/MPA/Fc-Py in 10 mM PBS (pH 7.4).

6. Differential pulse voltammograms (DPVs) obtained at CFME/Au/MPA/Fc-Py and Au/MPA/Fc-Py (Figure S7)

![Graph showing DPVs](image)

*Figure S7.* DPVs obtained at (a) Au/MPA/Fc-Py and (b) CFME/Au/MPA/Fc-Py in 10 mM PBS (pH 7.4).
7. Selectivity test (Figure S8)

Figure S8. (A) Selectivity against metal ions: (a) 0.5 pH, (b) K+, (c) Na+, (d) Ca2+, (e) Mg2+, (f) Cu2+, (g) Co2+, (h) Zn2+, (i) Ni2+, (j) Cd2+, (k) Mn2+, (l) Fe2+ and (m) Fe3+ (1 mM for b to e, 10 μM for f to m); (B) Selectivity against amino acids: (a) 0.5 pH, (b) Arg, (c) Cys, (d) Glu, (e) Gly, (f) His, (g) Leu, (h) Iso, (i) Lys, (j) Met, (k) Phe, (l) Ser, (m) Thr, and (n) Val (10 μM for b to n); (C) Selectivity against ROS: (a) 0.5 pH, (b) ROO•, (c) O2•–, (d) ClO•, (e) ONOO–, (f) H2O2, (g) 1O2, (h) •OH (10 μM for b to h); (D) Selectivity against other biological molecules: (a) 0.5 pH, (b) glucose, (c) 3-MT, (d) HVA, (e) DL-lactic acid, (f) L-tyrosine, (g) UA, (h) tyramine, (i) AA, (j) DOPAC, (k) DA, (l) 5-HIAA, (m) ATP (1mM for b, 10 μM for c to m).
8. Stability and Reproducibility test (Figure S9-S10)

**Figure S9.** Stability test for CFME/Au/MPA/Fc-Py in 10 mM PBS (pH 7.4) over 7 days.

**Figure S10.** Reproducibility test for CFME/Au/MPA/Fc-Py in 10 mM PBS (pH 7.4).
9. The pH values determined in different regions of rat brains upon global cerebral ischemia with different times (Figure S11)

**Figure S11.** The pH changes obtained from (A) hippocampus, (B) striatum, (C) cortex of rat brain upon different cerebral ischemia time.
**Table S1.** The pH values determined by the present pH biosensor compared with those obtained by fluorescent method in microdialysates.

<table>
<thead>
<tr>
<th>Region</th>
<th>Normal rat brain</th>
<th>Microdialysates</th>
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<tbody>
<tr>
<td></td>
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<td>pH</td>
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<tr>
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