An Electrochemical Biosensor with Dual Signal Outputs: Toward Simultaneous Quantification of pH and O₂ in the Brain upon Ischemia and in a Tumor during Cancer Starvation Therapy

Li Liu+, Fan Zhao+, Wei Liu, Tong Zhu, John Z. H. Zhang, Chen Chen, Zhihui Dai,* Huisheng Peng, Jun-Long Huang, Qin Hu, Wenbo Bu, and Yang Tian*

ange_201705615_sm_miscellaneous_information.pdf
### Contents

1. Experimental section
2. MS of Hemin-Fc molecule (Figure S1)
3. FT-IR spectra (Figure S2)
4. UV-visible spectra (Figure S3)
5. Stability of biosensor (Figure S4)
6. CVs obtained at Hemin-Fc/CNF microelectrode for different pH (Figure S5)
7. CVs obtained at Hemin-Fc/CNF microelectrode for different concentrations of O$_2$ (Figure S6)
8. Selectivity and competition test (Figure S7)
9. Results of pH and O$_2$ values obtained in rat brain upon carotid artery ischemia (CAI) model (Figure S8)
10. Results of pH and O$_2$ values obtained in mice brain upon MCAO and reperfusion (Figure S9)
11. TTC staining of ischemic brain slices (Figure S10)
12. TEM images of Mg$_2$Si nanoparticles (Figure S11)
13. CVs obtained at Hemin-Fc/CNT microelectrode in live rat tumor (Figure S12)
14. The concentration of O$_2$ and pH changes obtained from rat tumor at different times after injection of Mg$_2$Si nanoparticles (Figure S13)
15. Time-dependent depth profiles of the intratumoral O$_2$ concentrations and pH levels (Figure S14)
16. Representative HE-stained tumor tissue (Figure S15)
17. Elemental analysis (Table S1)

### 1. Experimental section

**Materials and Reagents.** Hemin and dimethyl sulfoxide (DMSO) were purchased from Aladdin-Reagent Company (Shanghai, China). Ascorbic Acid (AA), dopamine (DA), 5-hydroxytryptamine (5-HT), dihydroxy-phenyl aceticacid (DOPAC), glucose, uric acid (UA), potassium superoxide (KO$_2$), and 30% H$_2$O$_2$ were purchase from Sigma-Aldrich (USA). Aminoferrocene (Fc-NH$_2$) was obtained from Tokyo Chemical Industry Co. Ltd. (TCI). Chloroform, N, N - dimethyl aniline, and N,N'-Dicyclohexylcarbodiimide were bought from Shanghai Titan Scientific Co. Ltd. Disodium hydrogen phosphate (Na$_2$HPO$_4$), Sodium dihydrogen phosphate (NaH$_2$PO$_4$), sodium hydroxide (NaOH), sulfuric acid (H$_2$SO$_4$), Magnesium powder (200 mesh, 99%), silicon powder (200 mesh, 99.9%), ammonium hydroxide (NH$_3$•H$_2$O) and PVP.
(average relative molecular mass of 30,000 (PVP30)) and other chemicals were purchased from Sinopharm Chemical Reagent Co. Ltd. In selectivity experiments, superoxide anion (O$_2^-$) was root in dissolved KO$_2$ (150 μM) in the DMSO solution. Alkyl peroxy radical (ROO•) was chemically generated by thermolysis of AAPH (150 μM) in air-saturated aqueous solution at 310 K. Hypochlorite anion (ClO•) was provided by NaClO (150 μM). Nitroxy (HNO) was generated from solution of Angeli’s salt (10 μM). Nitric oxide (NO) was derived from the solution of diethylamine NONOate sodium salt (10 μM). Hydroxyl radical (•OH) was derived from Fenton’s regent (10 μM). Peroxynitrite (ONOO•) was chemically generated by the reaction between H$_2$O$_2$ (10 μM) and NaNO$_2$ (10 μM). The first singlet oxygen (1O$_2$) was produced by H$_2$O$_2$ with NaClO. All the solutions of metal ions were prepared from their chloride salts, which were obtained from Aladdin reagent. All reagents and chemicals were of analytical grade and were used without further purification. In this work, 0.1 M phosphate buffer saline (PBS) with various pH values were prepared by mixing stock standard solutions of 0.1 M Na$_2$HPO$_4$ and 0.1 M KH$_2$PO$_4$. pH was adjusted and controlled by pH meter (PHSJ-3F). Double-distilled water obtained from a Millipore water purification system (Milli-Q, ≥18.2 MΩ•cm) was used in all experiments and were at room temperature.

**Apparatus and Instruments.** The concentration of O$_2$ in solution was measured by Dissolved Oxygen Meters (JENCO 9173 DO). The mass spectrum was obtained from Agilent 1290 Infinity LC/6460 QQQ MS. The SEM image was taken by scanning electron microscope (JSM-5610LV, NORAN-VANTAGE). The TEM image was obtained by transmission electron microscope (Jem2100F, NORAN 6720A-3nes-sn). The transmission electron microscopy were acquired by High Resolution Transmission Electron Microscopy (HRTEM, JEOL JEM- 2010F, 200kV). Ultraviolet spectrum was performed on UV-VIS spectrophotometer (UH5300, Japan). Infrared spectrums (IR) were obtained by Infrared spectrometer (Thermo Scientific Nicolet iS5). The Raman spectrums of CNF were obtained by Raman spectrometer (Thermo Scientific DXRxi). Elemental analysis was carried out on an Elemental analyzer.
ARIO EL III. Electrochemical experiment was performed on an electrochemical work station (CHI660D, Chenhua in Shanghai, China) in 0.1 M PBS with a standard three-electrode system consisting of a reference electrode (Ag/AgCl), and a platinum (Pt) wire counter electrode.

Synthesis of Hemin-Fc. Firstly, chloroform (18 ml), hemin (257 mg, 0.3942 mmol), (9.5 mg, 0.078 mmol), N, N-dimethyl aniline, and (0.318 g, 1.58 mmol) were added into 100 ml flask successively with constant stirring under ice-bath. Then, chloroform (21 ml) and DDC (197 mg, 1.615 mmol) were dropped into the flask, which was reacted for 72 h under ice-bath. The reaction was monitored by thin-layer chromatography. After the reaction was completed, the mixture was washed with water for 3 times. Then, the organic layer was separated and dried by 5 g anhydrous sodium sulfate. Then 2 g silica gel was added and CHCl₃ was removed by rotary evaporation at room temperature. A black solid was separated by silica columns (0.2 g, 63% yield).

Synthesis of Mg₂Si nanoparticles. Mg₂Si nanoparticles were synthesized as previously reported. In brief, 40 mmol of silicon powder and 100 mmol of magnesium powder were mixed and placed in a 25 ml alumina crucible. Under an Ar/O₂ (5% O₂) atmosphere, the mixture was heated at 500 °C for 3 h with a ramping rate of 10 °C min⁻¹. The resultant product was immersed in 200 ml of 95% ethanol solution that contained 2 g of PVP 30 after cooled to room temperature, and then treated by ultrasonication at 60°C for 5 h to hydrate MgO adequately. The subsequent suspension was gently centrifuged at 5,000 revolutions per minute (r.p.m.) for 10 min to eliminate the insoluble Mg(OH)₂ and other large particles. The dispersed MS NPs were then collected by centrifugation at 13,000 r.p.m. for 15 min, and washed with ethanol three times.

Fabrication and Modification of Electrode. For preparation of carbon nanotube fiber
(CNF) microelectrode, multi-wall carbon nanotubes (MWNTs) were spun into 10 µm diameter bundles and then were heated for 2 h in the absence of O₂ in pipe type oven. The CNF tip was cut into ~10 mm length. Then, the CNF microelectrode was attached on a copper wire with silver paste and then dried. Next, it was carefully inserted into a single borosilicate glass capillary, sealing with epoxy resin and drying (60°C, 8h). For modification of CNF microelectrode with the as-synthesized Hemin-Fc, CNF microelectrode was immersed into the Hemin-Fc DMSO solution for 1 h. The modified electrode was denoted as Hemin-Fc/CNF microelectrode.

Animal Experiment upon Carotid Artery Ischemia (CAI). The animal surgery is based on previous literature.²² In brief, in vivo experiment on Male Wistar rats (250~300 g) purchased from Shanghai Laboratory Animal Co. Ltd (Shanghai, China). Rats were anesthetized with Chloral hydrate (1g ml⁻¹ solution, 0.3ml / 100g, i. p.) and mounted in a stereotaxic apparatus (Beijing Tide-Gene Biotechnology Development Centre). Two holes were drilled with cranial drill on rat’s skull. A larger holes for working electrode and microinjection probe (CMA/110/111 Tub) and the other hole for counter electrode and reference electrode. The reference electrode was placed in electrical contact with the brain tissue through a salt bridge. The microelectrode and micro-dialysis probe were implanted in the dorsal hippocampus (AP = 5.0 mm, L = 5.0 mm from bregma, V = 2.5 mm from the surface of the skull) using standard stereotaxic procedures. Furthermore, the left striatum (AP = 0 mm, L= 2.5 mm anterior to bregma, and V=7.0 mm from the surface of skull) and the cortex (AP = 0.2 mm, L=5.6 mm from bregma, V=3.0 mm from the surface of skull) were also examined.

Surgery for carotid artery ischemia (CAI) was performed according to the previous literature.³³ Namely, both common carotid arteries were exposed and isolated from surrounding connective tissue, with special care not to damage the valgus or sympathetic nerves running close by, making nylon thread went through a midline cervical incision. Gravity of medium-sized forceps hemostatic pulled nylon
thread to induced global ischemia in which situation maintained at most 30 min. After the experiment, the wound was sutured and the rat can regain its health. Ischemia of other brain regions were the same as those described above. During the whole experiment, the rat's body temperature remained at about 37°C with a heating pad and supplements of chloral hydrate were given as required.

**Animal Experiment upon MCAO.** The animal model protocols were approved by the Institutional Animal Care and Use committees of Shanghai Jiao Tong University School of Medicine, Shanghai, China. Male C57 mouse (25-30 g) were purchased from the Medical College of Shanghai Jiao Tong University. The mice were raised with free access to food and water in a climate controlled vivarium with a 12 h light-dark cycle. Chloral hydrate (Aladdin-Reagent Company, Shanghai, China) was diluted into 10% aqueous solution for experimental animals’ anesthesia. An intraluminal monofilament was used for conduction of MCAO and the following reperfusion operation. Through the internal carotid artery (ICA), the occlusion of middle cerebral artery was performed. Firstly, pterygopalatine artery of the ICA was identified and occluded with an artery clamp. Then, a small incision was created on the right external carotid artery (ECA), by which a monofilament suture was inserted into the ICA for about ~10 mm from the bifurcation. During the process, the right common carotid artery was occluded and was perfused along with the pterygopalatine artery of the ICA at the end of the surgery. A thermal insulation pad (TC-1000, Man Pu Biotechnology Co. Ltd., Shanghai, China) was used to maintain the animals at a body temperature of 37°C during and after the surgery. A Doppler laser blood flow meter (Periflux 5010, Perimed, Stockholm, Sweden) was used to detect the occlusion and reperfusion condition. The microelectrode was implanted in the dorsal cortex (left = 2.5 mm, forward = 0.5 mm, depth = 2 mm) using standard stereotaxic procedures. Furthermore, the striatum (left = 2.5 mm, backward = 0.35 mm, depth = 3 mm) were also examined.

After 24h reperfusion, brains were collected for 2,3,5-Tri
phenyltetrazoliumchloride (TTC) staining. The brains were cut into 1 mm slices and then immersed in 2% TTC solution at 37°C for 30 min. Paraformaldehyde solution (4%) is used for fixation of stained slices before photographing with a high resolution digital camera (Canon EOS70D).

**Tumor Experiments.** All procedures involving animals were conducted with the approval of the Animal Ethics Committee in East China Normal University, China. Six-week-old female BALB/c mice (~20 g) were purchased from the Laboratory Animal Center of the Chinese Academy of Science. SH-SY5Y cells were selected as the cancer model. SH-SY5Y cells at a density of 2×10^7 cells/mouse were subcutaneously injected into flanks of the nude mice two weeks before the animal tests. Tumors were harvested after intratumorally injected with the Mg_2Si nanoparticles (1 M in 30 µl saline) for different time, then fixed in 10% neutral buffered formalin, processed routinely into paraffin, sectioned at 4 µm, stained with H&E and examined under a BX51 optical microscope.

2. **MS of Hemin-Fc molecule**

![Figure S1. MS of Hemin-Fc molecule.](image-url)
3. FT-IR spectra

![FT-IR spectra](image)

*Figure S2.* FT-IR spectra of (a) Fc-NH₂, (b) Hemin, and (c) Hemin-Fc.

4. UV-visible spectra

![UV-visible spectra](image)

*Figure S3.* UV-visible spectra of (I) Hemin-Fc and (II) Hemin-Fc/CNF suspension. The solvent is TMF.
5. Stability of the present biosensor

![Graph](image)

**Figure S4.** (a) CVs obtained at Hemin-Fc/CNF microelectrode in N₂-saturated 0.1 M PBS (pH=7.4). Scan rate: 0.1 V s⁻¹. (I) the first cycle and (II) the 1000th cycle. (b) The stability of Hemin-Fc/CNF microelectrode stored in PBS solution at 0°C for 7 days.
6. CVs obtained at Hemin-Fc/CNF microelectrode for different pH
Figure S5. CVs obtained at Hemin-Fc/CNF microelectrode in 0.1 M PBS with different pH, bubbled with pure O₂ for different time. Scan rate: 0.1 V s⁻¹. Inset: The linear relationship of J_p/J_p⁰ with different concentrations of O₂.

(a) pH =5.0; (I) 1.56 μM, (II) 13.1 μM, (III) 25.6 μM, (IV) 39.1 μM, (V) 52.8 μM, (VI) 66.6 μM, (VII) 81.9 μM, (VIII) 98.1 μM, (IX) 115.0 μM, (X) 139.4 μM, (XI) 163.7 μM, (XII) 189 μM.

(b) pH=5.4; (I) 1.66 μM, (II) 12.5 μM, (III) 25.9 μM, (IV) 39.1 μM, (V) 50.0 μM, (VI) 67.5 μM, (VII) 83.4 μM, (VIII) 99.7 μM, (IX) 114.7 μM, (X) 131.3 μM, (XI) 150.6 μM, (XII) 167.8 μM.

(c) pH=5.8; (I) 1.87 μM, (II) 15.6 μM, (III) 29.4 μM, (IV) 46.2 μM, (V) 65.6 μM, (VI) 82.8 μM, (VII) 100.9 μM, (VIII) 118.1 μM, (IX) 154.4 μM, (X) 179.4 μM, (XI) 201.3 μM.

(d) pH=6.2; (I) 1.88 μM, (II) 18.4 μM, (III) 40 μM, (IV) 63.7 μM, (V) 81.2 μM, (VI) 100.0 μM, (VII) is 117.8 μM, (VIII) 137.8 μM, (IX) 158.1 μM, (X) 179.4 μM, (XI) 201.3 μM.

(e) pH=6.6; (I) 1.88 μM, (II) 15.9 μM, (III) 39.25 μM, (IV) 56.25 μM, (V) 80.75 μM, (VI) 100.63 μM, (VII) 119.1 μM, (VIII) 137.5 μM, (IX) 155.9 μM, (X) 172.5 μM, (XI) 190.6 μM.

(f) pH=7.0; (I) 1.31 μM, (II) 21.9 μM, (III) 35.9 μM, (IV) 53.4 μM, (V) 73.1 μM, (VI) 96.3 μM, (VII) is 113.1 μM, (VIII) 132.8 μM, (IX) 154.1 μM, (X) 180.6 μM, (XI) 206.9 μM.

(g) pH=7.8; (I) 2.1 μM, (II) 14.4 μM, (III) 28.4 μM, (IV) 42.8 μM, (V) 57.8 μM, (VI) 74.1 μM, (VII) 86.9 μM, (VIII) 112.5 μM, (IX) 133.1 μM, (X) 150.9 μM, (XI) 170.7 μM, (XII) 198.3 μM.

(h) pH=8.2; (I) 1.65 μM, (II) 14.7 μM, (III) 27.2 μM, (IV) 41.6 μM, (V) 57.5 μM, (VI) 72.2 μM, (VII) 88.8 μM, (VIII) 104.4 μM, (IX) 122.5 μM, (X) 138.7 μM, (XI) 157.5 μM, (XII) 172.8 μM.
7. CVs obtained at Hemin-Fc/CNF microelectrode for different concentrations of O$_2$

![Diagram of CVs at different O$_2$ concentrations and pH values]

**Figure S6.** CVs obtained at Hemin-Fc/CNT electrode in 0.1 M PBS for different concentrations of O$_2$. Scan rate: 0.1 V s$^{-1}$. Inset: The linear relationship of $\Delta E_{1/2}$ with different pH. (a) **concentration of O$_2$: 25 μM.** pH (I) 5.4, (II) 6.0, (III) 6.4, (IV) 6.8, (V) 7.0, (VI) 7.4, (VII) 7.8, (VIII) 8.1. (b) **concentration of O$_2$: 60 μM.** pH (I) 5.5, (II) 6.0, (III) 6.4, (IV) 6.8, (V) 7.0, (VI) 7.4, (VII) 7.8, (VIII) 8.0. (c) **concentration of O$_2$: 100 μM.** pH (I) 5.5, (II) 6.0, (III) 6.4, (IV) 6.8, (V) 7.0, (VI) 7.4, (VII) 7.8, (VIII) 8.0. (d) **concentration of O$_2$: 170 μM.** pH (I) 5.5, (II) 6.0, (III) 6.4, (IV) 6.8, (V) 7.0, (VI) 7.4, (VII) 7.8, (VIII) 8.1. Scan rate: 0.1 V s$^{-1}$. 
8. Selectivity and competition tests

**Figure S7.** (a and e) (a) Selectivity (Green bars) and competition (Pink bars) tests for neurotransmitters against O₂ detection, and (e) Selectivity test for neurotransmitters against pH determination. (1) 400 μM AA; (2) 10 μM UA; (3) 10 μM 5-HT; (4) 10 μM DA; (5) 10 μM DOPAC; (6) 1 mM glucose; and (7) 1 mM O₂.

(b) Selectivity (Green bars) and competition (Pink bars) tests for metal ions against O₂ detection, and (f) Selectivity test for metal ions against pH determination. (1) 1 mM K⁺; (2) 10 μM Ca²⁺; (3) 1 mM Na⁺; (4) 10 μM Mg²⁺; (5) 10 μM Cu²⁺; (6) 10 μM Fe³⁺; (7) 10 μM Fe²⁺; and (8) 1 mM O₂.

(c and g) (c) Selectivity (Green bars) and competition (Pink bars) tests for ROS against O₂ detection, and (g) Selectivity test for ROS against pH determination. (1) 1 μM H₂O₂; (2) 10 μM O₂⁻; (3) 10 μM ROO⁻; (4) 10 μM NO₂⁻; (5) 10 μM ONOO⁻; (6) 10 μM •OH; (7) 10 μM ClO⁻; (8) 10 μM NO; (9) 10 μM ¹O₂; (10) 10 μM HNO; (11) 1 mM O₂.

(d and h) (d) Selectivity (Green bars) and competition (Pink bars) tests for amino acids against O₂ detection, and (e) Selectivity test for amino acids against pH determination. (1) Asp; (2) Gly; (3) Leu; (4) Thr; (5) Cys; (6) D-Try; (7) Ser; (8) L-Arg; (9) His; (10) Lys; (11) Gln; (12) Met; (13) Val; (14) Ile; (15) 1 mM O₂.
9. Results of pH and $O_2$ values obtained in rat brain upon carotid artery ischemia (CAI) model

**Figure S8.** (a) CVs obtained at Hemin-Fc/CNF microelectrode in the striatum of (I) normal rat brain and (II-VIII) that followed by ischemia for (II) 3 min, (III) 6 min, (IV) 9 min, (V) 12 min, (VI) 15 min, (VII) 18 min, and (VIII) 21 min. (b) CVs obtained at Hemin-Fc/CNF microelectrode in the striatum of (I) normal rat brain and (II) that followed by ischemia for 21 min, and then (VIII) after reperfusion for 1 h. (c and d) The concentrations of $O_2$ (c) and pH values (d) in cortex, striatum, and hippocampus in rat brain followed by cerebral ischemia with different times.
10. Results of pH and O$_2$ values obtained in mice brain upon MCAO and reperfusion

![Bar graphs showing pH and O$_2$ concentrations in cortex and striatum](image)

**Figure S9.** (a and c) pH values and (b, and d) concentrations of O$_2$ obtained in cortex and striatum in live mice brain upon MCAO (a and b) for 0.5 h and followed by reperfusion, (c and d) for 1.5 h and followed by reperfusion.

11. TTC staining of ischemic brain slices

<table>
<thead>
<tr>
<th>MCAO duration</th>
<th>0</th>
<th>0.5</th>
<th>1.0</th>
<th>1.5</th>
<th>2.0 (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTC staining</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>after 24h</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure S10.** TTC-staining of ischemic brain slices after MCAO for 0, 0.5, 1, and 1.5 h followed by 24 h reperfusion. Neurons were lethal upon MCAO for 2 h.
12. TEM images of Mg$_2$Si nanoparticles

![TEM images of Mg$_2$Si nanoparticles](image)

**Figure S11.** TEM images of (a) highly dispersed Mg$_2$Si nanoparticles and (b) representative Mg$_2$Si nanoparticle.

13. CVs obtained at Hemin-Fc/CNT microelectrode in live mice tumor

![CVs obtained at Hemin-Fc/CNT microelectrode](image)

**Figure S12.** CVs obtained at Hemin-Fc/CNT microelectrode in live mice tumor before (I) and after Mg$_2$Si nanoparticles intratumorally injected into the tumor for (II) 0.5 h, (III) 1 h, (IV) 1.5 h, (V) 2 h, and (VI) 24 h.
14. The concentration of O₂ and pH changes obtained from mice tumor after injection of Mg₂Si nanoparticles

Figure S13. The concentration of (a) O₂ and (b) pH changes obtained from mice tumor at different times after injection of Mg₂Si nanoparticles.

15. Time-dependent depth profiles of the intratumoral O₂ concentrations and pH levels

Figure S14. Time-dependent depth profiles of the intratumoral (a) O₂ concentrations and (b) pH levels.
16. Representative HE-stained tumor tissue

![Representative HE-stained tumor tissue](image)

**Figure S15.** Representative HE-stained sections of SH-SY5Y xenografted tumor tissue collected at different times after injection of Mg$_2$Si nanoparticles.

17. Elemental analysis of H, C, and N in Hemin-Fc

<table>
<thead>
<tr>
<th>Table SI</th>
<th>Elemental analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experimental value</td>
</tr>
<tr>
<td>H</td>
<td>4.392</td>
</tr>
<tr>
<td>C</td>
<td>64.351</td>
</tr>
<tr>
<td>N</td>
<td>8.217</td>
</tr>
</tbody>
</table>

References:


(S4) J. Li; W. Liu; S. Ding; W. Xu; Y. Guan; J. H. Zhang; X. Sun, *Brain Res.* **2008**, 1210, 223-229.