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A Novel Electrochemical Biosensor with Dual Signal Outputs: Toward Simultaneous Quantification of pH and O_2 in Brain upon Ischemia and in Tumor during Cancer Starvation Therapy

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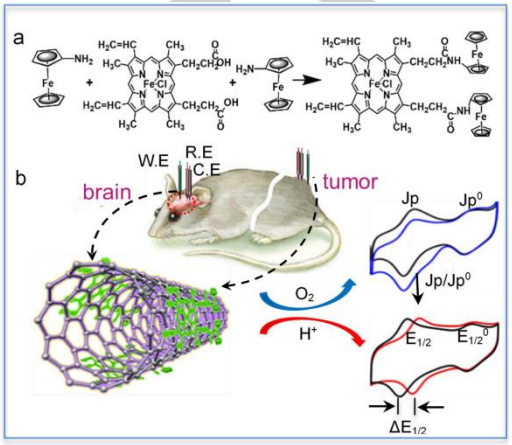
Abstract: In this work, we develop a novel methodology for designing electrochemical biosensors with both current and potential signal outputs for simultaneous determination of two biological species in the live system using one designed single molecule. Oxygen (O_2) and pH, simple and very important species, are employed as model molecules. By designing and synthesizing of a new molecule, Hemin-aminoferrocene (Hemin-Fc), we create a single electrochemical biosensor for simultaneous determination and ratiometric quantification of O_2 and pH in brain upon ischemia and in tumor during cancer starvation therapy. The reduction peak current of Hemin group increases with rising concentrations of O_2 from 1.3 to 200.6 μM. Meanwhile, this peak potential positively shifts with decreasing pH from 8.0 to 5.5, resulting in simultaneous determination of O_2 and pH by monitoring both current and potential signal outputs. Interestingly, Fc group can serve as an inner reference to construct a ratiometric biosensor, because both current and potential signals of Fc stay almost constant with variations of O_2 and pH. The developed single biosensor with high temporal and spatial resolutions, as well as remarkable selectivity and accuracy, is successfully applied in real-time quantification of O_2 and pH in brain upon ischemia, as well as in tumor during cancer therapy.

In vivo analysis of chemical signals in brain extracellular fluid (ECF) using implanted electrochemical biosensors is a vital way to study brain functions and brain activity mapping.[1] It offers excellent spatial (10-200 μm) and temporal (~s) resolutions, and a major advantage of long-term stability. By implanting a microelectrode in a specific brain region, changes in the concentration of a variety of ECF chemical species can be monitored through applying a suitable electrical signal and usually recording the resulting Faradaic current.[2] In this work, a novel methodology for designing electrochemical biosensors was created. Namely, one organic molecule Hemin-Fc was designed and synthesized for simultaneous recognition and real-time quantification of two model molecules, O_2 and pH, during brain ischemia as well as cancer therapy, by monitoring both current and potential signal outputs. Meanwhile, Fc functional group served as an inner reference for providing built-in correct, resulting in ratiometric determination with high accuracy.



Scheme 1. (a) Formation of conjugate Hemin-Fc from hemin and Fc. (b) Illustration for working mechanism of the present biosensor for simultaneous determination of O_2 and pH.

Both O_2 and pH are critically important species correlated with brain ischemia and cancer growth.[3] During ischemia, when O_2 supply is limited, the electron transport chain of the inner mitochondrial membrane becomes high reduced. In this reduced state, reactive oxygen species (ROS) production may result. Then, when respiration is inhibited but glycolysis persists, protons and lactate may generate, leading to pH decreasing.[4] On the other hand, both O_2 and pH are vital for cancer growth and the lack of O_2 can lead to hypoxia-induced cell death.[5] However, an analytical method is still the bottleneck for understanding the mechanism of ischemic brain damage, as well as that of cancer growth and death. Over the past decades, many elegant approaches have been established for monitoring of either O_2 or pH.[6] Our group is very interested in development of biosensors for detection of ROS, pH, and metal ions in the live systems.[7] But, it is still a challenging work to simultaneous recognition and accurate determination of two kinds of important species such as O_2 and pH in the live systems.

Herein, we designed and synthesized an organic molecule Hemin-Fc, in which Hemin group was connected to two aminoferrocene molecules by amide bond (Scheme 1a). As demonstrated in Scheme 1b, Hemin-Fc showed two pairs of well-separated electrochemical peaks: one pair located at ~ -356 mV vs. Ag/AgCl (half-wave potential, $E_{1/2}$) was ascribed to the redox reaction of $Fe^{2+/3+}$ in Hemin; the other one ($E_{1/2}$) observed at ~ 230 mV was attributed to that of Fc. The reduction peak current of $Fe^{2+/3+}$ clearly increased with the increasing concentration of O_2 because of electrocatalytic activity of Hemin

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group toward O_2. Interestingly, $E_{1/2}$ of $Fe^{2+/3+}$ in Hemin also shifted positively with decreasing pH from 8.0 to 5.5, resulting in simultaneous determination of O_2 and pH by monitoring both current and potential signal outputs. Moreover, Fc group provided a built-in correction for quantification of O_2 and pH, because neither peak current nor potential of Fc stayed almost constant toward changes of O_2 and pH. Meanwhile, carbon nanotube fibre (CNF) with a diameter of 10 μm provided a stable and biocompatible substrate for immobilizing Hemin-Fc through π-π stacking. Finally, the developed biosensor was successfully applied for simultaneous detection and accurate quantification of O_2 and pH in live animals brain upon ischemia. The accurate values of O_2 and pH were first reported in different regions of live brain upon ischemia, as well as in tumor during Mg_2Si-starved cancer therapy.

First of all, Hemin-Fc molecule was synthesized according to the procedure (Scheme 1a), and the structure was confirmed by

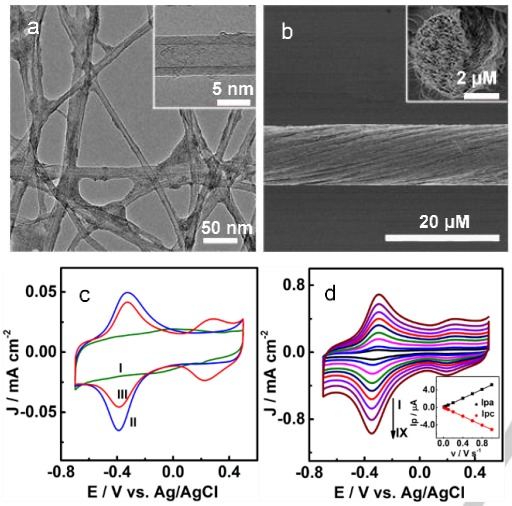

Figure 1. (a) TEM images of MWNTs; (b) SEM images of CNF. (c) Cyclic voltammograms (CVs) obtained in N_2-saturated 0.1 M PBS (pH =7.4), at (I) bare CNF, (II) Hemin/CNF, and (III) Hemin-Fc/CNF microelectrodes. Scan rate: 0.1 V s^{-1}. (d) CVs obtained at Hemin-Fc/CNF microelectrode in N_2-saturated 0.1 M PBS (pH=7.4) at different scan rates: (I) 0.01, (II) 0.02, (III) 0.05, (IV) 0.1, (V) 0.2, (VI) 0.4, (VII) 0.6, (VIII) 0.8, and (IX) 1.0 V s^{-1}. Inset: The linear relationship between peak current intensity of $Fe^{2+/3+}$ in Hemin and scan rate.

Mass spectroscopy (Figure S1, Supporting Information (SI)) and elemental analysis (Table S1, SI). Furthermore, a strong band at 1700 cm^{-1} observed for infrared spectrum of hemin chloride shifts to 1641 cm^{-1} in that of Hemin-Fc (Figure S2, SI), which was also evident that the carboxylic group in hemin chloride was successfully converted into an amide group (Scheme 1a).[8] On the other hand, multi-walled carbon nanotube (MWNT) fiber was dry-spun from spinnable MWNT arrays at a rotating rate of 2000 rpm, which were synthesized by chemical vapor deposition in advance.[9] The as-prepared MWCNTs are straight and less constraint from the environment (Figure 1a). The diameter of CNF is ~10 μm. The end of the MWCNT fiber is abundant with tips of the nanotubes, as given in Figure 1b. Then, Hemin-Fc molecule was attached on the CNF microelectrode through π-π stacking interaction, which was confirmed by UV-vis absorption spectroscopy (Figure S3, SI).[10] The CNF microelectrode assembled by Hemin-Fc molecules was denoted as Hemin-Fc/CNF microelectrode hereafter.

Electrochemical behavior of Hemin-Fc/CNF microelectrode was then investigated. As shown in Figure 1c, no obvious redox peak was observed at bare CNF microelectrode (Curve I), while a couple of peaks was obtained at Hemin-modified CNF (denoted as Hemin/CNF) microelectrode (Curve II) with $E_{1/2}$ of -356 ± 3 mV, ascribed to redox reaction of $Fe^{2+/3+}$ in Hemin on the CNF microelectrode. For Hemin-Fc/CNF microelectrode, as expected, two pairs of redox peaks were clearly observed, in which one pair located at -356 ± 3 mV ($E_{1/2}$) agreed well with the redox reaction of $Fe^{2+/3+}$ in Hemin; the other one ($E_{1/2}$) observed at 230 ± 2 mV was attributed to that of Fc group in Hemin-Fc molecule assembled on the CNF microelectrode. Furthermore, both anodic and cathodic peak current intensities of $Fe^{2+/3+}$ and Fc increased with increasing scan rates, as demonstrated in Figure 1d, and showed good linearity with scan rates. The result indicates the redox reaction of Hemin-Fc molecule on CNF microelectrode was surface-controlled process. The rate constant of the heterogeneous electron transfer (k_s) for Hemin on CNF microelectrode was estimated to be ~11.83 ± 0.08 s^{-1}, which is much greater than that obtained at carbon fiber microelectrode modified MWNT.[11] In addition, the redox responses of Hemin-Fc/CNF microelectrode demonstrated negligible change (< 2.7%) after this electrode was continuously scanned in PBS solution for 1000 cycles (Figure S4a, SI), revealing that Hemin-Fc molecule was stably assembled on CNF microelectrode through π-π stacking interaction. This Hemin-Fc/CNF microelectrode also showed negligible decrease after stored in PBS (pH 7.4) for 7 days (Figure S4b, SI). This characteristic is very beneficial for determination of biological species in the complicated live systems.

Next, the designed Hemin-Fc/CNF microelectrode was investigated toward determination of O_2 and pH. As demonstrated in Figure 2a, with rising concentration of O_2, the reduction peak current intensity (J_p) of Hemin gradually increased, which was resulted from the electrocatalytic activity of Hemin to convert O_2 into H_2O through electrochemical reaction followed by chemical reaction with one electron-one proton.[12] However, the reduction peak current intensity (J_p^0) for Fc stayed almost constant, leading to ratiometric determination (J_p/J_p^0) of O_2 with a linear range of 1.3-200.6 μM (Inset of Figure 2a). On the other hand, as shown in Figure 2b, $E_{1/2}$ of $Fe^{2+/3+}$ in Hemin shifted positively with decreasing pH, while that of Fc had negligible changes. This result allowed us determine pH with good linearity from 5.5 to 8.0 using the potential difference ($\Delta E_{1/2}$ between $E_{1/2}$ of $Fe^{2+/3+}$ and that of Fc. As plotted in inset of Figure 2b, the slope of ΔE versus pH was estimated to be -55.6 mV/pH, indicating Nernstian process of this redox reaction. More importantly, the developed Hemin-Fc/CNF biosensor can be applied to simultaneous detection of O_2 and pH. As shown in Figure 2c, J_p of $Fe^{2+/3+}$ in Hemin increased with increasing concentration of O_2 as well as $E_{1/2}$ shifted negatively with increasing pH. Interestingly, neither J_p nor $E_{1/2}$ of Fc showed obvious changes toward the variations of O_2 and pH.

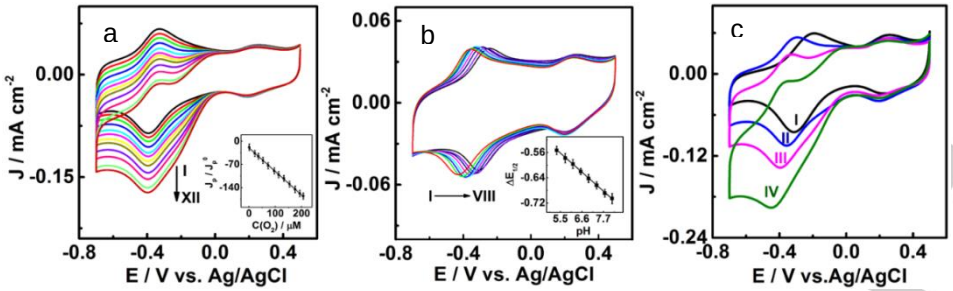

Figure 2. (a) CVs obtained at Hemin-Fc/CNF microelectrode in 0.1 M PBS (pH=7.4) bubbled with pure O_2 with different concentrations: (I) 1.3 μM, (II) 21.9 μM, (III) 35.9 μM, (IV) 53.4 μM, (V) 73.1 μM, (VI) 96.2 μM, (VII) 113.1 μM; (VIII) 132.8 μM, (IX) 154.1 μM, (X) 175.6 μM, (XI) 193.1 μM, and (XII) 206.9 μM. Inset: Calibration plot of J_p/J_p^0 against concentrations of O_2. (b) CVs obtained at Hemin-Fc/CNT microelectrode in 0.1 M PBS (N_2-saturated) with different pH values: (I) 8.14, (II) 7.77, (III) 7.35, (IV) 6.94, (V) 6.54, (VI) 6.14, (VII) 5.75, and (VIII) 5.31. Inset: The calibration plot of $\Delta E_{1/2}$ against different pH. (c) CVs obtained at Hemin-Fc/CNT microelectrode in 0.1 M PBS with different pH values and different concentrations of O_2. (I) pH=5.0, $C(O_2)$=1.6 μM; (II) at pH=6.6, $C(O_2)$=83.7 μM; (III) pH=7.4,$C(O_2)$=132.8 μM ; (IV) pH=8.2, $C(O_2)$=157.5 μM. Scan rate: 0.1 V s^{-1}.

Furthermore, Cubic spline interpolation was used to smooth the experimental measurements (Figure S5 & S6, SI). The resolution of potential, current, pH are improved to 0.001 V, 0.01 μA and 0.025, respectively. The calculation was performed using an in-house written computer program based on the functions SPLINE and SPLINT.[13] The relationship images were summarized in Figure 3, which were employed to quantify the levels of O_2 and pH in the live systems.

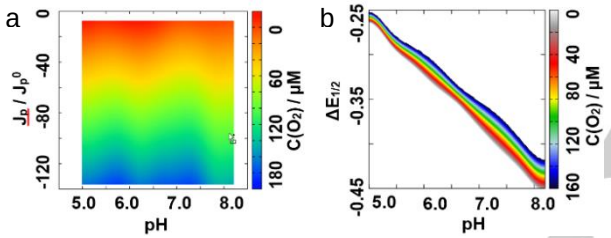

Figure 3. (a) Relational graph among J_p/J_p^0, the concentration of O_2, and pH; (b) Relational graph among $\Delta E_{1/2}$, the concentration of O_2, and pH.

Besides accuracy, selectivity is also relatively important for biosensors available for use in brain. The selectivity and competition test of the develop biosensor for simultaneous determination of O_2 and pH was examined against ROS, amino acids, and neurotransmitters. No obvious changes (< 4.8%) in both peak current and potential of $Fe^{2+/3+}$ were obtained for these biological species (Figure S7, SI). Meanwhile, negligible changes (< 3.2%) were observed for determination of O_2 and pH against the potential interferences. All the results indicate high selectivity of the present biosensor for simultaneous determination of O_2 and pH over other biological species which may coexist in the live systems.

As demonstrated above, the present biosensor shows high temporal and spatial resolutions with remarkable selectivity and accuracy. Thus, it was first applied in simultaneous determination of O_2 and pH in live brain. Two kinds of ischemic animal models were used: one is middle cerebral artery occlusion (MCAO),[14] another is carotid artery ligation (CAI) (Figure S8, SI).[15] Figure 4 shows CVs obtained at Hemin-Fc/CNF microelectrode in striatum of normal mouse brain and that followed by MCAO. It is clear that the reduction peak of $Fe^{2+/3+}$ gradually decreased and peak potential positively shifted with MCAO time prolonged. After reperfusion, both peak current and potential returned to almost background (Figure 4b) . It is worth mentioning that the redox peak of Fc had almost no changes in either current or potential, confirming that Fc can be used as an inner reference. Moreover, the concentrations of O_2, as well as pH values in cortex, upon MCAO with different times were also investigated (Figure S9, SI). As demonstrated in Figure 4c, pH value was observed to 7.21±0.05 and 7.25±0.05 in cortex and striatum of normal mouse brain, and remarkably went down to 6.87±0.04 and 6.78±0.04 after MCAO time lasted up to 1.5 h. The initial concentration of O_2 was estimated to 61.0±6.8

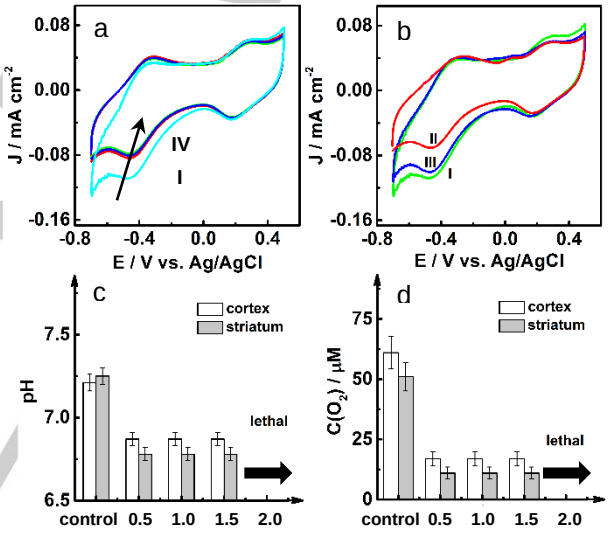

Figure 4. (a) CVs obtained at Hemin-Fc/CNF microelectrode in the striatum of (I) normal mouse brain and (II-IV) that followed by MCAO for (II) 0.5 h, (III) 1 h, and (IV) 1.5 h. (b) CVs obtained at Hemin-Fc/CNF microelectrode in the striatum of (I) normal mouse brain and (II) that followed by MCAO for 1.5 h, and then (III) after reperfusion for 1 h. (c) pH values and (d) concentrations of O_2 obtained in cortex and striatum in live mice brain upon MCAO for 0.5, 1.0, 1.5, and 2 h; neuronal cells were lethal upon 2 h MCAO.

and 51.0±5.9 μM in cortex and striatum (Figure 4d), and gradually decreased down to 17.0±2.8 and 11.0±2.5 μM after MCAO duration time prolonged up to 1.5 h. As MCAO time increased to 2 h, neuronal cells were lethal. More interestingly, TTC (2, 3, 5-triphenyltetrazolium chloride) staining was employed for the fast and reliable visualization of hypoxic and ischemic brain tissue.[16] With the ischemic duration prolonged, the size of cerebral infarction gradually increased (Figure S10, SI). After MCAO duration for 2 h, the long-time ischemia induced neuronal death and ischemic brain damage. The present study provided the direct evidence that the severity of neuronal death strongly depends on the increased length of brain acidity that lasts for ~2.0 h.

Furthermore, our uesful tool was successfully expanded to real-time track O_2 concentrations and pH levels in tumor of live

mice after Mg_2Si nanoparticles injected for cancer starvation therapy (Figure S11-S14, SI).[17] The initial concentration of O_2 in tumor was estimated to 136.6±9.7 μM, and drastically dropped to 53±5.1 μM after injection of Mg_2Si nanoparticles for 1 h. After 2 h, the concentration of O_2 decreased to 4.8±1.3 μM and remained stable even till 24 h. On the other hand, pH value in tumor increased sharply from 6.46±0.04 to 7.08±0.05 within 1 h, then gradually went up to 7.18±0.05 after 2 h and maintained a steady state afterwards. Experiments were also carried out in different regions and depths of mouse tumors, which are similar to those demonstrated above. Pathological haematoxylin and eosin (H&E) staining assays also showed that after 1 h the tumors starved by the deoxygenation agent feature obvious and significant fibrosis, necrosis and apoptosis, and such damage became substantially more serious over 24 h (Figure S15, SI). These results for the first time demonstrated valuable and accurate information on the concentrations of O_2 and pH levels in tumor of live mice upon cancer starvation therapy. Using our useful tool, this work gave the direct evidence that the formation of products from Mg_2Si nanoparticles in tumor environment blocks blood capillaries and prevent tumors from receiving new supplies of O_2 and nutrients.

A single biosensor for simultaneous recognition and accurate quantification of O_2 and pH using both current and potential signal outputs with quick response was developed through designing and synthesizing one molecule – Hemin-Fc, which was further assembled onto biocompatible CNF surface with small size down to ~10 μm. The redox peak of Fc provided a built-in corrects for avoiding environmental effects, leading to high accuracy of this biosensor. Besides this, the present biosensor demonstrated high spatial and temporal resolution, with remarkable selectivity and long-term stability. According, the developed biosensor was successfully applied in simultaneous detection of O_2 and pH in live brain followed by ischemia, as well as in tumor during cancer starvation therapy. The present work have not only provided a new methodology to designing biosensors for simultaneous determination of multi-species using different signal outputs, but have established a reliable approach for obtaining important information of biological species, which should be very valuable for understanding physiological and pathological pathway of diseases.

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Keywords: Brain chemistry • Cancer therapy • Analytical Methods • oxygen • pH

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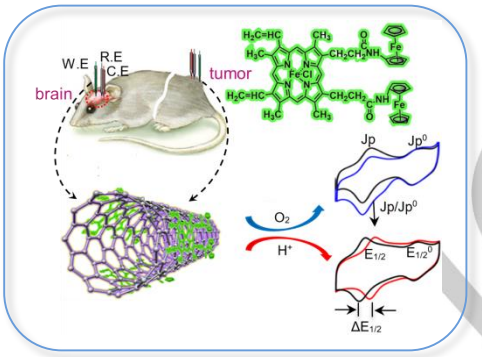
COMMUNICATION

In this work, a novel methodology for designing electrochemical biosensors was created, by monitoring both current and potential signal outputs. One organic molecule Hemin-Fc was designed for simultaneous recognition and ratiometric quantification of two model molecules, O_2 and pH. The developed single biosensor with high temporal and spatial resolutions was successfully applied in real-time quantification with high accuracy in rat brain upon ischemia and in tumor during cancer starvation therapy.



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