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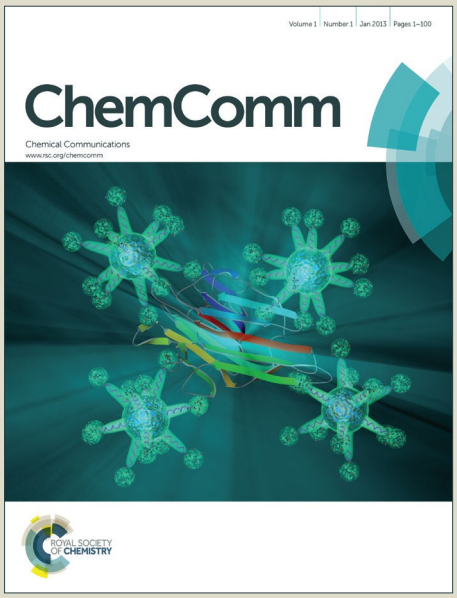




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# In Vivo Monitoring of Local pH Values in a Live Rat Brain Based on Design of Specific Electroacitve Molecule for $H^+$

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**pH plays an important role in the biochemical, ion-regulatory, or electrical machinery of nerve and glial cells, and is considered to be related to lots of degenrative diseases. Herein, we first develop a two-channel electrochemial ratiometric biosensor for local pH determination in a live rat brain, and report the accurent pH values in the different regions of live brains upon by global cerebral ischemia.**

Local pH of brain microenvironment plays a significant role in an amount of physiological activities, including signal transduction, cell growth and apoptosis, enzymatic activities and ion transportation.[1] Even a slight change in pH can cause apparent effects on the biochemical, ion-regulatory, or electrical machinery of nerve and glial cells, furthermore, brain acidosis will augment cell death under various pathological conditions.[2] Moreover, pH has been considered to be closely correlated to many chronic degenerative diseases, such as Parkinson's disease, Alzheimer's disease and ischemia stroke.[3] Within seconds of initiation of global brain ischemia, consciousness is lost, and if sustained for as little as 5 min, severe brain damage is induced.[3c] Stroke is one of the leading causes of death and disability humans.[3d] However, the detailed mechanism is still unclear, mainly because a lack of accurate analytical methods to monitoring of local concentrations of biological species, e.g. pH, in the live brain.

In the past decades, several techniques have been developed for pH monitoring, including nuclear magnetic resonance (NMR), UV-visible absorption method, fluorescence spectroscopy and electrochemical methods.[4,5] Fluorescence spectroscopy has attracted much attention from different research fields, because it is very powerful in biosensing and bioimaging in tissues and live cells with high spatial and temporal resolution.[4] Our group has developed the fluorescent probes for monitoring pH gradients in live cells and tissues.[4a,b] However, the method is very difficult to real-time measurement of pH in the live rat brain. Electrochemical approaches provided notable advantages in sensitivity and simplicity, especially easy to miniaturization for real-time monitoring in the live systems.[5] Ion-Sensitive Microelectrodes (ISMEs) are often employed to detect pH in live cells and tissues.[5b] Unfortunately, ISME made from glass is easy to be broken and very hard to be miniaturized for in vivo determination. Despite several electrochemical pH determination approaches have been established, only a few papers have reported in vivo detection of pH in live animals.[6] The pioneering work by Wightman's group through fast-scan cyclic voltammetry opened an elegant way to characterize the local pH changes in brain.[5b-d] However, there are still several challenges for real-time detection of pH in a live rat brain, ranging in selectivity against metal ions, reactive oxygen species (ROS), and other biological species, as well as accuracy in response to the complicated environment of live rat brain. Finally, the biosensor is necessary to be easily miniaturized in order to limit the damage in the rat brain.

In this work, we designed and synthesized N-(6-aminopyridin-2-yl)ferrocene (Fc-Py), with specific recognition element for pH – pyridine (Py) and electroactive element – ferrocene (Fc), as illustrated in Scheme 1, as an electrochemical probe for determination of pH. The anodic and cathodic potentials of Fc-Py shifted with the pH changes, resulting in a pH-sensitive electrode with a linear range from 5.9 to 8.0. The developed Fc-Py with reversible redox behavior specifically responded to $H^+$, and showed high selectivity over metal ions including $Na^+$, $K^+$, $Ca^{2+}$, $Mg^{2+}$, ROS, amino acids, neurotransmitters, and other biological species. Meanwhile, 6-(ferrocenyl)hexanethiol (FcHT) with a separated redox potential, inert to pH, served as an inner reference channel to provide a built-in correct for avoiding the complicated brain environment, thus resulting in a two-channel ratiometric electrochemical pH sensor. In addition, the current signal was

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*† Electronic Supplementary Information (ESI) available: experimental section; synthetic route and NMR spectra of Fc-Py; XPS spectra for different modified electrodes; DPV response of CFME/Au/MPA/Fc-Py; selectivity and stability test; pH value in different region upon different cerebral ischemic time. See DOI: 10.1039/x0xx00000x*

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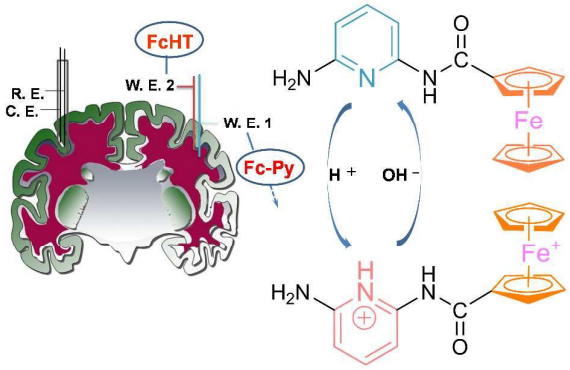


**Scheme 1.** Illustration of two-channel electrochemical biosensor for in vivo monitoring of pH in a live rat brain.

amplified by ~6-fold through the electrodeposited gold bamboo shoot-like nanostructures on carbon fiber microelectrode (CFME) with diameter of 10 µm. The sensitivity of the biosensor was greatly enhanced and the detection limit was achieved down to 0.13 pH. Finally, the developed biosensor for pH with high analytical performance and unique properties of CFME such as easy to be miniaturized and good biocompatibility, made it successful for real-time monitoring of pH values in the live rat brain followed by ischemia. We found that the microenvironment in striatum and hippocampus of rat brain changed into more acidic, and pH value reduced by ~0.53, and ~0.51, respectively, when the rat was aroused by global cerebral ischemia. However, negligible pH change was found in cortex of rat brain even upon ischemia. To the best of our knowledge, it is the first report for the accurate pH values in the different regions of live rat brains upon global cerebral ischemia.

First of all, Fc-Py was designed and synthesized according to the procedure (Fig. S1, ESI†), and characterized by NMR (Fig. S2 and Fig. S3, ESI†) and MS (Fig. S4, ESI†). Meanwhile, gold bamboo shoot-like nanostructures were electrodeposited on the CFME surface, which was denoted as CFME/Au. From the typical SEM images (Fig. 1), bamboo shoot-like gold structures full of nanoparticles were uniformly distributed on CFME surface. When we took a close look, it was found the length of the gold nanostructures is ~1-3 µm (Fig. 1B and 1C). From the XRD pattern (Fig. 1D), three typical peaks corresponding to (111), (200), (220) diffraction peaks were clearly observed, suggesting that the gold nanostructures are composed of crystalline gold.[7a] The modification of MPA and Fc-Py on CFME/Au electrode was tracked by X-ray spectroscopy (XPS, Fig. S5, ESI†). After gold nanostructures were deposited on CFME, obvious peaks located at 83.7 eV and 87.3 eV for Au $4f_{7/2}$ and Au $4f_{5/2}$ (curve b, Fig. S5A) were obtained.[7b] The presence of $S_{2p}$ (curve c, Fig. S5B) peak located at 165.2 eV indicates the successful modification of MPA on CFME/Au through Au-S bond, which was denoted as CFME/Au/MPA.[7b] The observation of 400.8 eV for $N_{1s}$ (curve d, Fig. S5C), 713.8 eV and 727.6 eV for Fe $2p_{3/2}$ and Fe $2p_{1/2}$ (curve d, Fig. S5D), revealing the immobilization of Fc-Py molecules on the

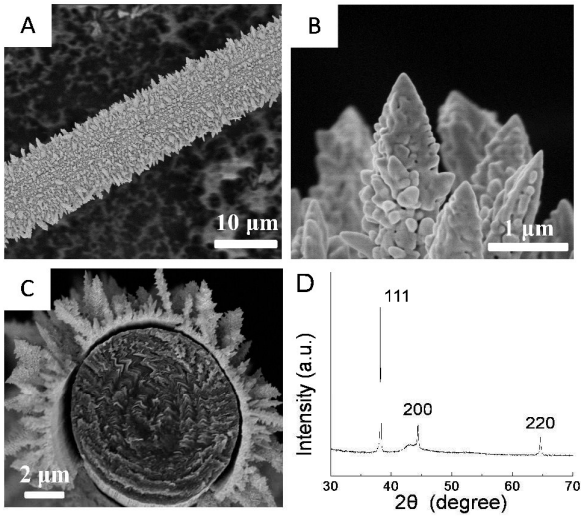


**Figure1.** SEM images of (A) CFME/Au, (B) the enlarged bamboo shoot-like gold nanostructures on CFME surface, (C) the cross section of CFME/Au (C). (D) XRD pattern of CFME/Au.

CFME/Au/MPA electrode.[7a] This Fc-Py-functionalized electrode was defined as CFME/Au/MPA/Fc-Py.

Because of its high sensitivity, differential pulse voltammetry (DPV) was employed in our electrochemical experiments. As shown in Fig. 2, anodic and cathodic peaks were clearly observed at 120 mV and 150 mV vs. Ag/AgCl at the CFME/Au/MPA/Fc-Py electrode in 10 mM PBS solution (pH 7.4), while no responses were obtained at other modified electrodes (Fig. S6, ESI†). This redox peak was attributed to ferrocene group of Fc-Py molecule. The mid-point potential $E_{1/2}$ of Fc-Py ($E_{1/2}$(Fc-Py)) positively shifted with the decreasing pH (Channel 1). This observation is possibly due to the increase in the electron-withdrawing power of the pyridine ring upon protonation of the pyridyl nitrogen atom as the pH decreased.[8] Thus, the ferrocene moiety became harder to be oxidized and the $E_{1/2}$(Fc-Py) positively shifted. On the other hand, the mid-point potential $E_{1/2}$ of FcHT ($E_{1/2}$(FcHT)) stayed almost constant (Channel 2), resulting in two-channel ratiometric determination of pH values. The potential separation ($\Delta E_{1/2}$ = $E_{1/2}$(FcHT) - $E_{1/2}$(Fc-Py)) between $E_{1/2}$(FcHT) and $E_{1/2}$(Fc-Py) shows a good linearity with pH ranging from 5.9 to 8.0. The detection limit was achieved down to 0.13 pH (3S/N). The pKa value of Fc-Py in aqueous media obtained by Henderson-Hasselbach-type mass action equation was estimated to be ~3.71. When Fc-Py was immobilized on microelectrode, the pKa value changed to be ~5.95. The current response obtained at CFME/Au/MPA/Fc-Py was improved by ~6.0-fold compared with that obtained at the Au/MPA/Fc-Py without electrodeposited Au nanostructures because of the amplified property of the nanostructured gold (Fig. S7, ESI†).

The complexity of the brain system presents a significant challenge for analytical performance not only in sensitivity and accuracy, but more importantly in selectivity. The selectivity of the present method was examined by monitoring $\Delta E_{1/2}$ in 10 mM PBS caused by potential interferences including metal ions, such as $Na^+$,

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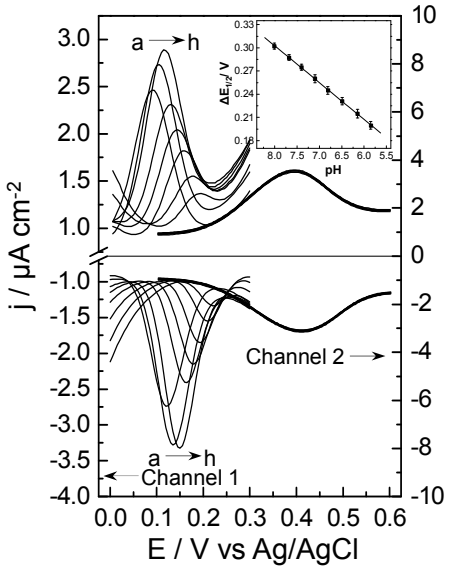


**Figure 2.** DPV responses of CFME/Au/MPA/Fc-Py (channel 1) and CFME/Au/FcHT (channel 2) electrodes in 10 mM PBS with pH changes: (a) 8.0, (b) 7.7, (c) 7.4, (d) 7.1, (e) 6.8, (f) 6.5, (g) 6.2, (h) 5.9. The inset shows the calibration plot of $\Delta E_{1/2}$ against pH.

$K^+$, $Ca^{2+}$, $Mg^{2+}$, $Cu^{2+}$, $Zn^{2+}$, $Co^{2+}$, $Ni^{2+}$, $Cd^{2+}$, $Mn^{2+}$, $Fe^{2+}$ and $Fe^{3+}$, ROS such as superoxide anion ($O_2^{\bullet-}$), alkyl peroxyl radical ($ROO^{\bullet}$), hypochlorite anion ($ClO^-$), peroxynitrite ($ONOO^-$), hydrogen peroxide ($H_2O_2$), singlet oxygen ($^1O_2$), and hydroxyl radical ($^{\bullet}OH$), amino acids including L-arginine (Arg), L-cysteine (Cys), L-glutamine (Glu), glycine (Gly), L-histidine (His), L-leucine (Leu), L-isoleucine (Iso), L-lysine (Lys), L-methionine (Met), L-phenylalanine (Phe), L-serine (Ser), L-threonine (Thr), and L-valine (Val), neurotransmitters and other biological species such as D(+)-glucose, 3-methoxytyramine hydrochloride (3-MT), 5-hydroxyindole-3-acetic acid (5-HIAA), homovanillic acid (HVA), DL-lactic acid, adenosine 5'-triphosphate disodium salt hydrate (ATP), tyramine, L-tyrosine, uric acid (UA), ascorbic acid (AA), dopamine (DA), DL-mandelicacid (DOPAC). No obvious changes in $\Delta E_{1/2}$ were observed upon the addition of metal ions, ROS, amino acids, and other biomolecules, relative to pH change by 0.5 unit (Fig. S8, ESI†). These results demonstrated the high selectivity of our pH biosensor over biological species. We also tested the stability of the modified electrode (Fig. S9, ESI†). No obvious changes ($<$6.30 %) were obtained for $\Delta E_{1/2}$ upon continuous test over 7 days, suggesting the long-term stability of the developed biosensor for pH. $\Delta E_{1/2}$ also maintained almost constant for four different electrodes, revealing good reproducibility (Fig. S10, ESI†).

The developed two-channel ratiometric biosensor for pH with high analytical performance and good biocompatibility, provided an accurate and selective platform for in vivo monitoring of pH values in live rat brains. Typical DPV responses were recorded after implantation of CFME/Au/MPA/Fc-Py electrode in the striatum of live rat brain. Fig. 3 shows electrochemical responses for pH obtained at CFME/Au/MPA/Fc-Py in the normal brain (curve a) and in the rat brain followed by global cerebral ischemia (curve b). Two couples of clear anodic (Fig. 3A) and cathodic (Fig. 3B) peaks were observed for the CFME/Au/MPA/Fc-Py in the normal brain and in the rat brain followed by global cerebral ischemia. The basal level of pH in the striatum region of brain was estimated to be 7.11±0.05 on

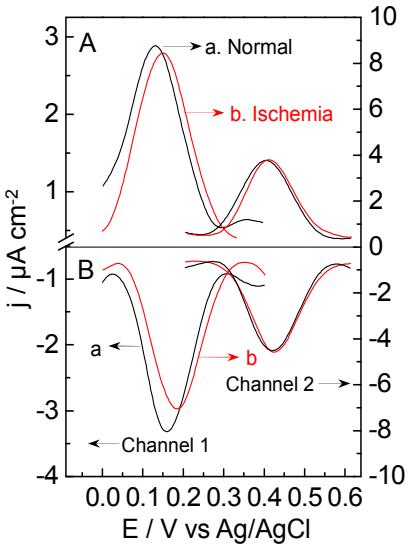


***Figure 3.*** DPVs obtained at the two-channel ratiometric electrochemical biosensor (channel 1: CFME/Au/MPA/Fc-Py; channel 2: CFME/Au/FcHT) for determination of pH in a rat brain: (a) black curves for normal rat brain and (b) red curves for rat brain followed by cerebral ischemia.

average, which is similar as the reported previously in rat tissues.[9] Moreover, $E_{1/2}$(Fc-Py)) positively shifted upon global ischemia (Channel 1), while $E_{1/2}$(FcHT) had negligible changes (Channel 2). Thus, $\Delta E_{1/2}$ became smaller upon ischemia, suggesting the microenvironment of striatum turned into more acidic (6.58±0.06) due to the cerebral ischemia.

Similar experiments were also carried out in different regions of rat brains, including striatum, hippocampus and cortex under different times. The determined pH values were concluded in Table 1. Obvious pH decreasing was clearly obtained in hippocampus (~0.51) and striatum (~0.53) of rat brains, respectively. But negligible pH change was obtained in cortex region of rat brain upon global cerebral ischemia. The pH values determined by our pH biosensor demonstrated a good agreement with those obtained by fluorescence spectroscopy using fluorescein isothiocyanate (FITC) as a probe (Table S1, ESI†). The pH changes were also recorded after cerebral ischemia for different times (Fig. S11, ESI†). These results first provided valuable and accurate information of pH values in live rat brains upon global cerebral ischemia.

To sum up, we have first developed a selective and accurate two-channel electrochemical biosensor for ratiometric monitoring of pH values in a wide range. On one hand, we have synthesized a specific recognition molecule for $H^+$, Fc-Py, for selective determination of pH over metal ions, ROS, amino acids, and other biological species. On the other hand, we have employed an inner reference molecule – FcHT for constructing a two-channel ratiometric biosensor for pH detection to avoid the effects from complicated live rat brain. In addition, sensitivity of the present pH biosensor has been improved by ~6-fold through electrodeposition of bamboo shoot-like gold nanostructures, with a low detection limit of 0.13 pH. The remarkable analytical performance, together with the unique

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**Table 1:** pH determined by the present method in different regions of normal rat brains and rat brains followed by global cerebral ischemia.

| | Normal rat brain | | | | Ischemia | | | |
|---|---|---|---|---|---|---|---|---|
| **Region** | Rat 1 | Rat 2 | Rat 3 | Mean±SD (n=3) | Rat 1 | Rat 2 | Rat 3 | Mean±SD (n=3) |
| | | pH | | | | pH | | |
| **Hippocampus** | 7.13 | 7.02 | 7.15 | 7.10±0.06 | 6.57 | 6.61 | 6.60 | 6.59±0.02 |
| **Striatum** | 7.10 | 7.04 | 7.20 | 7.11±0.07 | 6.65 | 6.50 | 6.59 | 6.58±0.06 |
| **Cortex** | 7.05 | 7.13 | 7.13 | 7.07±0.04 | 7.14 | 7.02 | 7.06 | 7.07±0.10 |

properties of CFME, has provided a reliable platform for real-time determination of pH values in live rat brains followed by global cerebral ischemia. On the basis of the accurate measurements, we have first reported the accurate pH values in the different regions of live normal rat brains and those followed by ischemia, which should be very useful for understanding the exact role that pH plays in the physiological and pathological processes of brain events. This methodology can also be extended to design and develop biosensors for other biological species, and provides practicability for further research of brain chemistry.

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