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Advanced Electrochemical Strategy for *in Vivo* Detection of Electrochemically Inactive Molecules

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Abstract: Development of efficient electrochemical strategies for *in vivo* analysis of electrochemically inactive molecules in brain is significant for understanding and studying their molecular mechanism and roles playing in brain and brain diseases. This review gives a brief introduction on the advanced *in vivo* electrochemical sensor for detection of non-redox active molecules from three aspects: 1) The selection and design of specific molecules are highly desirable to develop electrochemical sensors with high selectivity for measuring electrochemical inactive molecules through converting specific chemical reaction involved by target to electric signal; 2) The analysis based on ion current rectification occurred at spatial confined micro-interface provides a promising alternative way to realize *in vivo* monitoring of chemical inert molecules. 3) Integration of electrochemical sensors onto electrode arrays and new concept of dual signal outputs establish a flexible and promising approach for the further analysis of multiple species. At last, some perspectives are highlighted in the further development of the efficient electrochemical platform for *in vivo* detection of electrochemical inactive ions.

Key words: electrochemically inactive; electrochemical strategy; multiple detection; *in vivo*; brain
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In vivo analysis of chemical signals is an essential way to investigate brain function and brain activity mapping[1-2]. Electrochemical methods have attracted increasing attention because they allow high spatial resolution, temporal resolution and convenient real-time sensing[3-7]. The changes in the concentration of a variety of extracellular fluid (ECF) chemical species can be monitored by applying a suitable electrical signal, usually recording the resulting Faradic current[8-11]. However, there are several species difficult to be detected directly according to their own Faradic signal due mainly to two reasons: firstly, their redox potential is beyond the potential window of water decomposition; secondly, their overpotential is too large to be observed in normal polarized window. These molecules are called as electrochemically inactive molecules.

In fact, many non-redox active molecules play critical roles in brain functions. For instance, since acid-base brain chemistry usually correlates closely with various brain activities and functions, slight pH variation normally relates to the occurrence of brain disease such as epilepsy, ischemia, and psychiatric disorders, and contributes significantly to morbidity and mortality through a number of proton-sensitive processes such as ion channel gating, synaptic transmission, and enzymatic activity in brain energy metabolism[12-13]. Nitric oxide (NO) diffuses to smooth muscle cells, and produces guanosine 3′, 5′-cyclic monophosphate (cGMP) by activating soluble guanylyl cyclase[14]. The increased cGMP activates potassium ion (K^+) channels, causing K^+ efflux and hyperpolarization of cell membrane. The NO/cGMP/K^+ channel pathway has been reported to be involved in forebrain cholinergic neuron excitability on which learning and memory are critically dependent[15]. Magnesium ion (Mg^{2+}) is the active center for the chlorophyll, maintaining metabolism of organism and catalysis of

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many bioenzymes[16-17]. Excess of Mg^{2+} can close certain types of calcium ion (Ca^{2+}) channels, decreasing nerve cells activity[18-21]. Glucose provides nearly exclusive metabolic energy for brain and may activate neurons in peripheral and central nervous systems, so the dysfunction of glucose metabolism could initiate widespread disease of the peripheral and central nervous systems, as well as cardiovascular disease, nephropathy, and retinopathy. Actually, decreased glucose metabolism usually has come up before the emergence of brain pathology and cognitive impairment[22-23]. Therefore, development of novel and efficient electrochemical approach for *in vivo* analysis of electrochemically inactive molecules in brain is significant for understanding and studying their molecular mechanism and roles playing in brain and brain diseases.

Aiming to the limitation on detection of electrochemically inactive molecules with Faradic current response, enzyme-based sensors have been widely developed for the selective detection of various electrochemically inactive molecule, such as $O_2^{\cdot-}$, glucose, lactate[24-25]. However, the number of available natural biocatalysts and recognition molecules is quiet limited. Therefore, the selection and design of specific molecules are highly desirable to develop electrochemical sensors with high selectivity for the measurement of electrochemical inactive molecules through converting specific chemical reaction involved by target to electric signal. In addition, classic sensing strategy at solid electrode/solution interface is very limited in dealing with chemical inert substances. The analysis based on ion current rectification occurred at spatial confined micro-interface provides a promising alternative way to realize the *in vivo* monitoring of more electrochemical inactive molecules[26]. In addition, in order to obtain more information in brain, it is a urgent demand to develop *in vivo* platform for multiple detection of targets. The integration of electrochemical sensors onto electrode arrays and new concept of dual signal outputs establish a flexible and promising approach for the further analysis of multiple species.

Taking some non-redox active species as typical instances, this review gives a brief summary on recent advances for *in vivo* monitoring of electrochemically inactive molecules through establishing functional surfaces (conventional flat electrode surface, and inner wall surface of pores) with selecting and designing specific recognition molecules, which have led to improvements of the selectivity, accuracy, and simultaneous detection of two species. Moreover, currently available electrochemical strategies still leave great chances for efficient detection of electrochemical inactive molecules with high selectivity, high accuracy, and ability for multi-detection. Thus, the last section outlines prospective directions for surface design and new model of signal readout toward the design of advanced devices with multiple targets.

1 More Reliable pH Monitoring in Rat Brains Based on the Nernst Equation

1.1 Monitoring pH Variation Using Membrane Coated Carbon Fiber Electrodes

Real-time detection of pH in central neuron system is very important in investigation on physiological and pathological processes[27-28]. In brain, pH fluctuation is restricted around 7.4 in healthy condition through the regulation by acid-base homeostasis [29]. Once pH values are out of the physiological range, the acid-base disturbance will occur. Slight changes in intracellular or extracellular pH can produce conspicuous effects on the biochemical, ion-regulatory, or electrical machinery of nerve and glial cells. Moreover, brain acidosis will augment cell death under various pathophysiological conditions, which might cause many chronic degenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease, and ischemia[30-32].

Glass electrode currently is the most commonly used electrode for pH because of highly selective and reliable measurements in a wide pH range[33]. However, it is easy to be broken and is very difficult to be miniaturized for *in vivo* measurement of pH. Despite

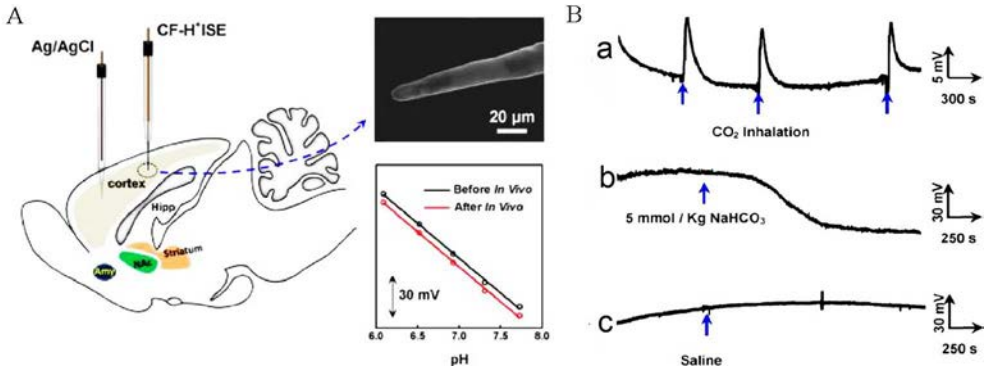

Fig. 1 (A) Schematic representation for *in vivo* monitoring of pH change in live brain of rats, with the SEM image of CF-H^+ISE, and the pH responses before and after *in vivo* implantations. (B) Potential responses recorded with the CF-H^+ISEs in amygdaloid nucleus (a), cerebral cortex (b, c) under different conditions of CO_2 inhalation (a) and intraperitoneal injection of $NaHCO_3$ (b) or saline (c).

many pH determination methods being reported, only a few works have realized *in vivo* monitoring of pH in live animals. Fast-scan cyclic voltammetry with carbon microelectrodes pioneered by Wightman's group provided an elegant way to characterize the local pH changes in the brain[34]. However, it still remains a challenge to develop an accurate biosensor applicable for real-time monitoring of pH values *in vivo* because of three main aspects: the resistance to the adsorption of protein in long-period detection, the high temporal/spatial resolution, and the high selectivity of the pH sensor.

To satisfy all the requirements mentioned above, Hao et al. developed an *in vivo* potentiometric method for quantitative monitoring pH changes in live rat brains using carbon fiber-based proton-selective electrodes (CF-H^+ISEs)[35]. The CF-H^+ISEs were fabricated by formation of a H^+-selective membrane with polyvinyl chloride polymeric matrixes containing plasticizer bis(2-ethylhexyl)sebacate, H^+ ionophore tridodecylamine, and ion exchanger potassium tetrakis (4-chlorophenyl)borate onto carbon fiber electrodes (Fig. 1A). The comparison of *in vitro* and *in vivo* results demonstrated that the CF-H^+ISEs exhibited good antifouling property against proteins. This character ensured the good sensitivity and reversibility for pH sensing during *in vivo* measurements. With the aid of this method, the acid-base disturbances in the brain acidosis induced by CO_2 inhalation and brain alkalosis induced by bicarbonate injections have been recorded (Fig. 1B). This study demonstrates a new potentiometric method for *in vivo* measurement of pH change in the live brain with high reliability.

1.2 Potential-Dependent Biosensor with an Internal Reference for Determining pH

The most popular pH determination strategy is based on the Nernst equation, which yields a well-defined linear correlation between the potential and pH. The ion-selective microelectrode coated by membrane with good anti-biofouling ability provides an efficient way to improve the accuracy of electrode. However, in contrast to the small solution resistance in a 0.1 mol·L^{-1} dilute electrolyte solution (~ 100 Ω), in the complicated brain environment the presences of many coexisting proteins and other biological species increase the solution resistance in cerebrospinal fluid up to 3000 ~ 5000 Ω[36]. As a result, the *IR* drop for a microelectrode with a current of ~1 μA will increase to 3 ~ 5 mV in the brain. This value is comparable to the change in potential induced by a pH change of 0.1 pH units for an electrochemical process with a linear variation of 59 mV·pH^{-1} unit. A pH variation as small as 0.2 ~ 0.5 pH units in living organisms has been attributed to a variety of severe cellular problems and diseases. Therefore, pH measurements based on potential variations without built-in corrections lead to a determination error of

20% ~ 50% for pH in the brain.

To overcome this difficulty, a two-channel electrochemical pH biosensor with an internal reference has been developed by Zhao et al., for the real-time detection of pH in different regions of rat brains upon ischemia (Fig. 2A)[37]. In one channel, Fc-Py is synthesized as an electrochemical probe for the high-selective recognition of pH. The ferrocene part is redox active in electrochemical path. Meanwhile, the pyridine part has response to the protons. In the other channel, FcHT with a separated redox potential is inert to pH, and hence utilized as an internal reference to promote the accuracy of pH sensor. The data of pH calibration curve is accumulated from the difference of the half-wave potential ($E_{1/2}$) between the response and the reference signal ($\Delta E_{1/2}$). The $\Delta E_{1/2}$ value is less influenced by the potential of outer reference electrode and the potential shift ascribes to *IR* drop. Therefore, the accuracy of pH monitoring *in vivo* would be greatly promoted. Nevertheless, the sensitivity of this system is 0.13 pH units, which is still too big for particularly recording small fluctuations of pH in living organism. Thus, a novel biosensor with one electrode is decorated with the specific recognition of 1,2-naphthoquinone (1,2-NQ) and the FcHT (CFME/Au/1,2-NQ+FcHT) (Fig. 2B). This sensor has a $2H^+$/2e approach in a wide pH range from 5.8 to 8.0, and the LOD has been successfully lowered to 0.07 pH units (Fig. 2C-2D)[38]. The detection results of pH in different brain regions of normal rats are 7.21 ± 0.05 in the striatum, 7.13 ± 0.09 in the hippocampus, and 7.27 ± 0.06 in the cortex, while after global cerebral ischemia, the pH values drop to 6.75 ± 0.07 in the striatum and 6.25 ± 0.03 in the hippocampus, along

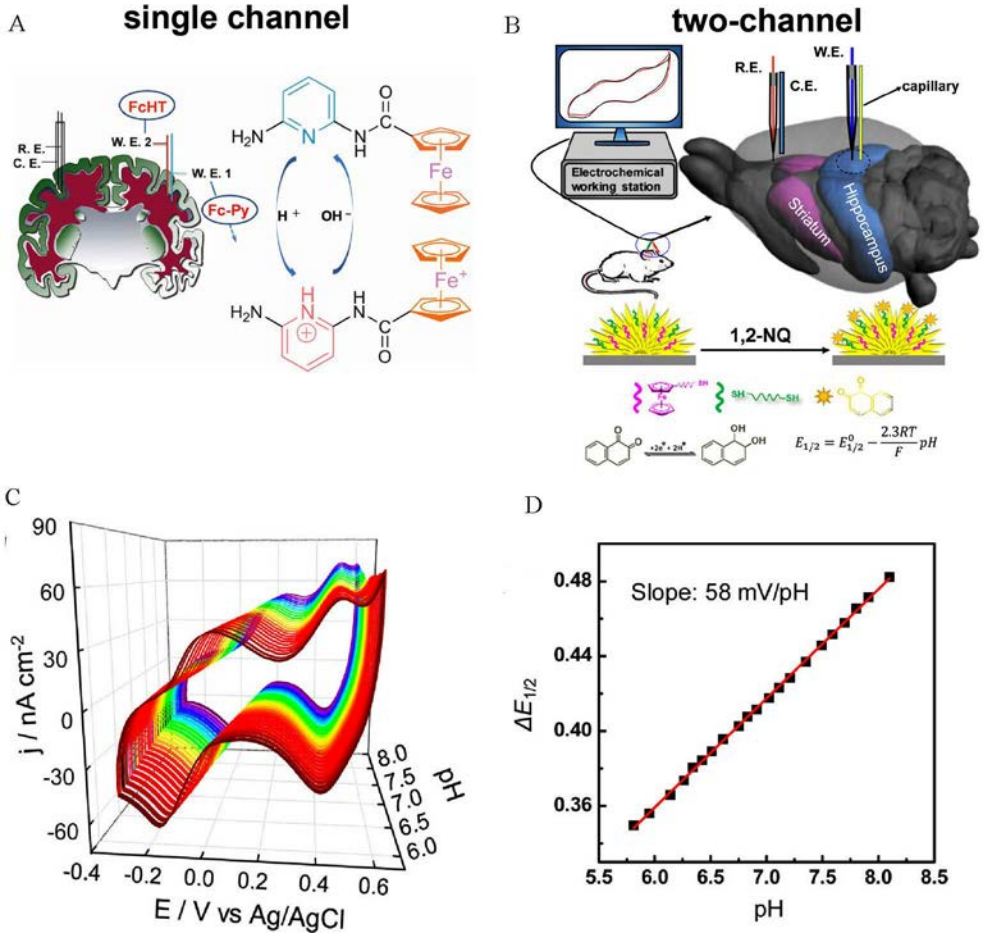

Fig. 2 pH sensor with single channel model (A) and two-channel model (B) for real-time monitoring of pH in a rat brain. CVs obtained at CFME/Au/1, 2-NQ+FcHT (C) and the corresponding $\Delta E_{1/2}$ calibration plot (D).

with a slight pH change in the cortex. This is the first report of the accurate pH values in different regions of normal rat brains and those after global cerebral ischemia, and may inspire new hypothesis of the influence of the pH on pathology processes in different brain regions.

With the similar pH strategy, 9, 10-anthraquinone was selected as a selective pH sensor, Liu et al. were devoted to developing a novel approach for simultaneous detection of Cu^+ and pH through rationally designing and synthesizing a series of N, N-bis(2-[2-(ethylthio)ethyl]-based derivatives as the specific recognition of Cu^+ (Fig. 3A-3D)[39]. The single electrochemical sensor can simultaneously determine Cu^+ concentrations from 0.5 to 9.5 μmol·L^{-1} and pH values from 6.0 to 8.0. Eventually, the efficient biosensor has been contributed to simultaneous detection of Cu^+ and pH in the live brain. It has been found that the concentrations of Cu^+ increased by 1.5-fold in cortex, 2.0-fold in striatum and hippocampus, in the mouse model of AD as compared to those of normal condition (Fig. 3E). Surprisingly, negligible pH change

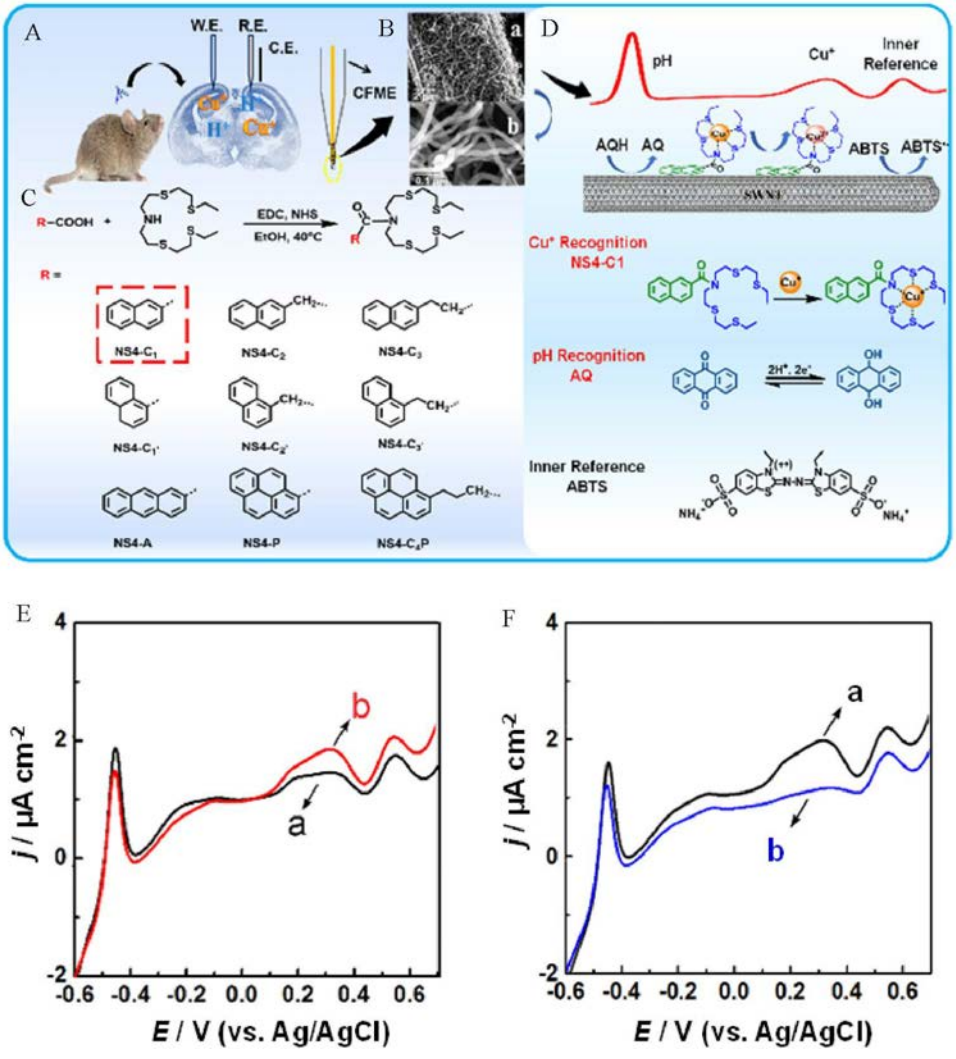

Fig. 3 (A) The developed ratiometric single biosensor for simultaneous evaluating the levels of Cu^+ and pH in a live mouse brain. (B) SEM images of (a) CFME/SWNT and (b) the enlarged image of SWNTs. (C) The general synthesis procedure of NS4s and the corresponding molecular structures. (D) Preparation procedures for the functionalized electrodes. (E) LSVs obtained at CFME/SWNT/AQ+NS4-C1+ABTS electrode in (a) normal mouse brain and (b) mouse brain with AD. (F) LSVs of CFME/SWNT/AQ+NS4-C1+ABTS electrode in (a) mouse brain with AD, and (b) followed by microinjection of 0.1 mmol·L^{-1} EDTA (pH 9.0).

has been observed. The increased Cu^{+} concentration observed in the mouse model of AD might be generated from the reduction of Cu^{+} by overexpressed amyloid precursor protein (APP). APP is involved in Cu homeostasis and processes a copper-binding site in its NH_2-terminal cysteine-rich domain, which can reduce Cu^{2+} to Cu^{+}.

2 *In Vivo* Analysis of Cerebral ATP with Micropipettes

Adenosine triphosphate (ATP) is one of the most important chemical signal molecules, playing an essential role in both energy metabolism and signal transduction[40-42]. Moreover, ATP has also been confirmed as a neurotransmitter correlated with the gustation even if its basal concentration in central nervous system (CNS) is relatively low (10^{-7} mol·L^{-1}·mg^{-1} tissue)[40]. However, ATP is an electroinactive molecule, and thus is challenging to be directly detected at a conventional bare electrode.

With the development of the microfabrication and micro/nano manufacture techniques, the new sensing principles based on nanochannel have attracted more and more attention for single molecule counting and biosensing. Two main signal output models have been conducted: one is based on the resistance-pulse technique, and the other is based on the ionic current rectification. Although the former one has been widely used for sensing single entity, it remains challenging for complex sample analysis since the tiny pore is easily blocked by the nontarget. In contrast, the later one based on a physical phenomenon of ion current rectification is susceptible to the surface chemistry of channel. When the target is passing through the channel, it would affect the surface chemistry and ion distribution in channel, and further change the current ration in the opposite potential. However, the relatively tiny and soft tip of nanopipettes renders difficulties in applying this method for real sample analysis in a simple way. To solve this challenge, Zhang developed an approach to selectively sensing ATP based on dual-recognition-units strategy[43]. Polyimidazolium brush is first modified onto the inner wall of micropipette by atom transfer radical polymerization to enable the occurrence of ion current rectification (ICR) obtained at a micrometer scale (Fig. 4). The presence of ATP leads to the dissociation of the ATP aptamer from the inner surface because it combines with the aptamer more strongly, resulting in the increase of net surface charge and thus the rectification ratio. The constructed ATP sensor exhibited good linearity ranging from 5 to 100 nmol·L^{-1}. Combining with *in vivo* microdialysis, the ATP sensors were used for cerebral ATP assay. This novel sensing platform paves a new way for *in vivo* analysis of electrochemically active species, and tissue-implantable *in vivo* analysis.

3 Multi-detection of Electrochemically Inactive Metal Ions Based on Functionalized Surface

3.1 Monitoring of Mg^{2+} and Ca^{2+} Based on NADH Oxidation Enhancement

Calcium ion (Ca^{2+}) is an important signal transduction element and is required for many functions including neurotransmitter release, and synaptic

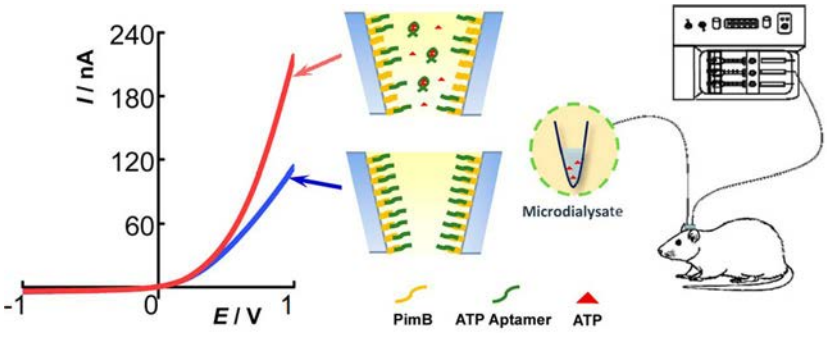

Fig. 4 Schematic illustration of MICR-based sensor for cerebral ATP assay combined with microdialysis technique.

transmission[18-20]. Meanwhile, magnesium ion (Mg^{2+}) is an important mediator and regulator of Ca^{2+} signaling and plays a classic role in defining the properties of ATP[16-17,21,43]. Excess of Mg^{2+} ions can close certain types of calcium channels, decreasing nerve cells activity[16-17]. Thus, monitoring concentrations of Mg^{2+} and Ca^{2+} at the same time is remarkably necessary. Unfortunately, though some biosensors have been developed for *in vitro* detection of Mg^{2+} and Ca^{2+}, simultaneous detection of Mg^{2+} and Ca^{2+} in central neuron system still remains a challenge.

Based on the enhancement of divalent cation toward the inner-sphere electrocatalytic oxidation of β-nicotinamide adenine dinucleotide (NADH), a new detecting system was developed for effective monitoring of electrochemically inactive Mg^{2+} and Ca^{2+}[44]. Toluidine blue O (TBO) was modified at electrode surface to detect Mg^{2+} and Ca^{2+} in brain dialysate of rats mixed with 1 mmol·L^{-1} NADH in Tris-HCl buffer (mixed ratio: 1:1). Besides, the same dialysate was also mixed with 1 mmol·L^{-1} NADH and 2 mmol·L^{-1} EGTA (as Ca^{2+}-chelating agent) in Tris-HCl buffer (mixed ratio: 1:1) to get specific enhancement of Mg^{2+}. By comparison of two sets of data to basic enhancement with different concentrations of Mg^{2+}/Ca^{2+}, the concentrations of Mg^{2+} and Ca^{2+} in dialysate can be both obtained. Taking advantage of this system, the basal concentrations of Mg^{2+} and Ca^{2+} in the dialysates from the rat cortex are calculated to be 285 ± 106 μmol·L^{-1} and 240 ± 92 μmol·L^{-1}, respectively. The results are quite close to those detected by traditional methods, such as ICP-AES. This highlights the reliability and accuracy of the system, implying the potential that systems with same principle may have good application in research of physiological and pathological processes.

3.2 Dual Electrochemical Microsensor for Simultaneous Monitoring of NO and K^+ in Brains

Nitric oxide (NO) has been confirmed to play an important role in a variety of biological/physiological events, such as neurotransmission, immune response, and so on[45-46]. Potassium ion (K^+) has been known as a fundamental factor in blood pressure regulation. There are some reports that salt-sensitive hypertension may be attenuated with K^+ supplementation by increasing NO production in Dahl rats[47]. Therefore, detailed analyses of NO and K^+ in living biological system are required to understand their functions. J. Moon et al. fabricated a dual electrochemical microsensor and intended for simultaneous detection of NO and K^+. Integration of two different transduction schemes (amperometry and potentiometry) in a single sensor body, a new two-electrode sensor has been developed[48]. One electrode (WE1) generates current proportional to NO concentration by the direct oxidation of NO; the other electrode (WE2) is an all-solid-state K^+ ISE whose junction potential correlates with K^+ activity at the membrane/electronic conductor interface (Fig. 4A). As shown in Fig. 4B-4C, typical dynamic responses of WE1 and WE2 to NO concentration, and those of WE1 and WE2 to K^+ concentration clearly indicate that no mutual interference occurs, confirming the feasibility of the detection system. What's more, the LOD of both electrode can cover the range of concentration of the target molecules (NO/K^+) in physiology and pathology processes, indicating that this system can record all the concentration changes of NO/K^+ in these processes. Employed with the good performance of the electrode, the newly developed NO/K^+ electrode was implanted in the cortex region of rat brain, and simultaneously measure the NO and K^+ concentrations under epileptic seizure. Preceding increase in NO level followed by a rather gradual increase in K^+ level is supposed seemingly to be that the measured ΔCNO and ΔCK^+ are related to cerebral vascular smooth muscle: the enhanced NO production in response to seizure mediates hyperpolarization, causing K^+ efflux from the smooth muscle cells. The concept through integrating two different transduction schemes (amperometry and potentiometry) on one sensor could be widely applied for *in vivo* detection.

3.3 Single Biosensor for Simultaneous Quantifications of Glucose and pH Using Both Current and Potential Outputs

Glucose provides nearly exclusive metabolic energy for brain and may activate neurons in peripheral

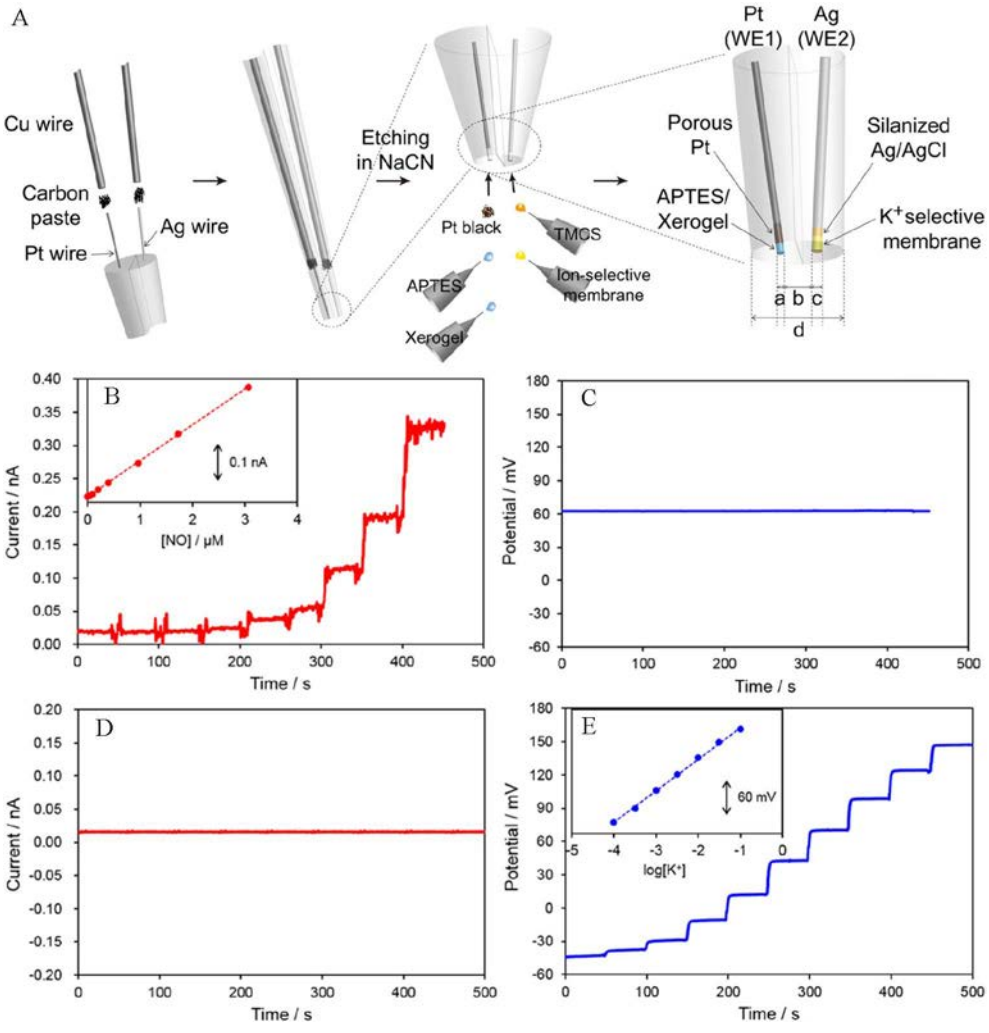

Fig. 5 (A) Schematic illustration for the preparation steps of an insertable NO/K^+ dual microsensor. The dual sensor has two working electrodes, WE1 and WE2. Typical dynamic response curves of (B) WE1 and (C) WE2 to NO concentration change; and those of (D) WE1 and (E) WE2 to K^+ concentration change.

and central nervous systems, so the dysfunction of glucose metabolism could initiate widespread disease of the peripheral and central nervous systems, as well as cardiovascular disease, nephropathy, and retinopathy[49-50]. Actually, decreased glucose metabolism usually has come up before the emergence of brain pathology and cognitive impairment[51-52]. Though the urgent need of diabetes diagnosis has tremendously arisen general interests in *in vitro* biosensing of glucose[53-54], the complicated cerebral environment under pathological conditions greatly blocks the detection of glucose. Glucose oxidase (GOD) has been widely employed in the design of glucose biosensor due to their specificity to glucose. The electrochemical response of GOD on electrodes originated from the redox reaction of flavine adenine dinucleotide (FAD) encapsulated in the enzyme molecule. FAD undergoes a two-electron coupled with two-proton redox reaction. Recently, Li et al. developed a single ratiometric biosensor taking direct electron transfer of GOD as a specific element for simultaneously quantifying the levels of pH and glucose using current and potential outputs in a live rat brain of diabetic model (Fig. 6A)[55]. Meanwhile, an insensitive molecule toward pH and glucose, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) is served as reference signal for providing built-in correction to avoid brain environmental effect. The ratio between the oxidation

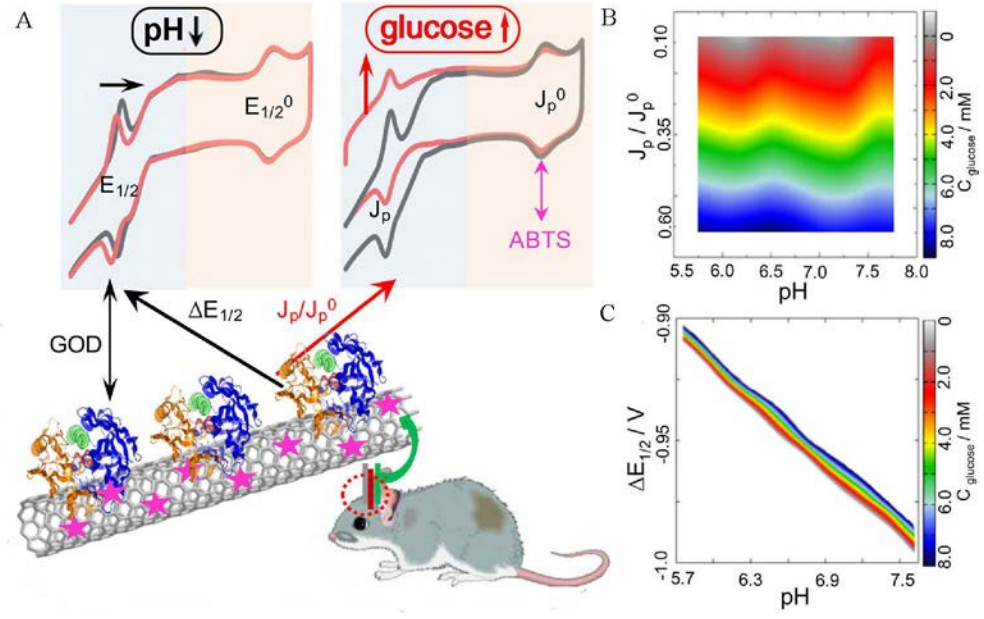

Fig. 6 (A) Developed ratiometric electrochemical biosensor for simultaneous detection and real-time quantification of pH and glucose sensor in a rat brain; (B) Relational graph among J_p/J_p^0, the concentration of glucose, and pH; (C) Relational graphs among $\Delta E_{1/2}$, the concentration of glucose, and pH.

peak current density of glucose and that of ABTS gradually increased with increasing concentration of glucose with a good linearity in the range of 0.3 ~ 8.2 nmol·L^{-1}. Meanwhile, the midpotential difference between GOD and ABTS positively shifted with pH decreasing, resulting in accurate determination of pH in the linear range of 5.67 ~ 7.65. Notably, cubic spline interpolation was used to smooth the experimental data. Relationship images were employed to quantify the levels of glucose and pH in live systems (Fig. 6B-6C). The detection data *in vivo* shows that the basal pH concentration decreased to 6.9 ± 0.1 in the striatum and 7.1 ± 0.1 in the cortex of rat brains of a diabetic model. The glucose concentrations are 2.22 ± 0.18 mmol·L^{-1} in the striatum and 1.44 ± 0.12 mmol·L^{-1} in the cortex of normal rat brain, in contrast to 4.53 ± 0.40 mmol·L^{-1} in the striatum and 6.65 ± 0.31 mmol·L^{-1} in the cortex in the brain of diabetic rat. Detailed information about the levels of pH and glucose in these rat brains may help us understand damage to brain function in diabetics. The simplicity of operation and instrumentation of the biosensor should promote its use in a broad range of biochemical applications.

4 Conclusions and Outlooks

The development of *in vivo* detection methods for electrochemically inactive molecules in brain chemistry is challenging but essential for advance in understanding the roles of the electrochemically inactive molecules playing in physiological and pathological processes. The design principles based on the efficient molecule design and new sensing model with micropipette greatly enriched the designing strategy for *in vivo* monitoring of electrochemically inactive molecules in brain. In addition, multiplex assays are urgent demands for comprehensive elucidation of brain science. The conventional way is using a unique code from a particular reporter to identify a specific target, but the resolution of acquired information is greatly limited by the number of distinguishable codes. The electrochemical voltammetric readouts are suffering from the limited potential window of water splitting (~ 1.5 V) and the signal overlapping resulting from wider Faradic current outputs (> 50 mV) upon multiple-label assay. In addition, combination of one probe for multi-detection with statistical method, for example, principal component analysis, might pave a promising way to simultaneous mea-

surement of multiple targets. On the other hand, liquid-liquid interface is taking as a promising platform for detection of more than one target at the same time. The use of pipet electrodes based on ionic transfer across an interface between two immiscible electrolyte solutions is an important model to determine ionic analytes which are non-redox active. In summary, currently available electrochemical strategies still leave great opportunities for the development of novel designs for efficient detection of electrochemical inactive molecules.

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非电活性分子的活体电化学分析进展

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摘要: 发展非电活性分子的活体电化学分析方法,对于解析这些物质在生理过程和病理过程中的作用具有重要研究意义. 本综述从三种分析策略出发,简要介绍了最近活体电化学传感器的研究进展:1)设计和筛选高选择性配体,通过将特异性的化学反应转换成电化学信号,发展了新型的非电活性分子的活体分析;2)利用微型孔道里的整流效应,结合特异性配体,建立了非电活性分子的新型分析平台;3)结合微电极阵列技术及同时分析多种输出信号的新型分析模式,实现活体中的多种非电活性物质的同时分析.

关键词: 非电活性分子;电化学传感;多分子分析;活体;脑