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A Single Biosensor for Evaluating the Levels of Copper Ion and L-Cysteine in a Live Rat Brain with Alzheimer's Disease

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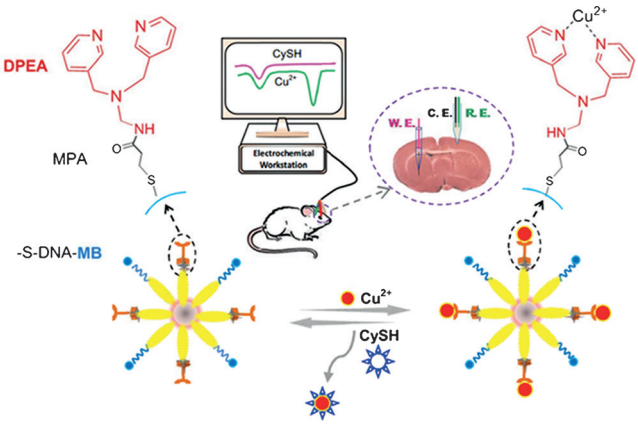
Abstract: *Copper ion (Cu^{2+}) and L-cysteine (CySH) are closely correlated with physiological and pathological events of Alzheimer's Disease (AD), however the detailed mechanism is still unclear, mainly owing to a lack of accurate analytical methods in live brains. Herein, we report a single biosensor for electrochemical ratiometric detection of Cu^{2+} and CySH in live rat brains with AD. N,N-di-(2-picoly)ethylenediamine (DPEA) is first synthesized for specific recognition of Cu^{2+} to form a DPEA–Cu^{2+} complex. This complex shows high selectivity for CySH owing to the release of Cu^{2+} from the complex through CySH binding to Cu^{2+} center. In parallel, 5′-MB-GGCGCGATTTTTTTTTTTT-SH-3′ (HS-DNA-MB, MB = Methylene Blue) is designed as an inner-reference for providing a built-in correction to improve the accuracy. As a result, combined with the amplified effect of Au nanoleaves, our single ratiometric biosensor can be successfully applied in real-time detection of Cu^{2+} and CySH in the live rat brains with AD. To our knowledge, this is the first report on the accurate concentrations of Cu^{2+} and CySH in live rat brains with AD.*

With the aging of the world population, Alzheimer's disease (AD) has become an increasing and serious public health problem in the industrialized world. Metal ions, as relatively vital messengers,[1] have been proposed to play significant role in the assembly and neurotoxicity of AD amyloid-β fibrils.[2] Many reports have indicated that metal ions are closely implicated in the pathogenesis of AD, especially Cu^{2+}.[3] Neurofibrillary tangles and senile plaques isolated from AD brains are capable of generating toxic reactive oxygen species (ROS) that depend on the presence of Cu^{2+} and Fe^{3+}.[4] Cu^{2+} ions are found concentrated within senile plaques of Alzheimer's disease patients directly bound to amyloid-β peptide (Aβ), and are linked to the neurotoxicity and oxidative stress.[5] On the other hand, L-cysteine (CySH), a thiol-containing amino acid, has also received great attention owing to the fact that abnormal concentration of CySH is considered to be signal for many brain diseases, such as AD and Parkinson's disease. However, the detailed mechanism is still unknown. Thus, the development of in vivo monitoring strategies of Cu^{2+} and CySH involved in brain chemistry is essential to progress our understanding of the roles that Cu^{2+} and CySH play in pathological and physiological events in the brain.

In the past decades, several elegant approaches have been established for detection of Cu^{2+} or CySH.[6–11] Electrochemical approaches show remarkable advantages in sensitivity and simplicity, especially for real-time measurements and in vivo determination.[12] We have developed a two-channel ratiometric biosensor for Cu^{2+} determination in a rat brain, but this sensor is suffering from the inaccurate detection from two different working electrodes.[11c] More importantly, few papers have been reported for detection of Cu^{2+} and CySH using a single biosensor. In this work, a single ratiometric biosensor was first designed and developed for determination of Cu^{2+} and CySH with high selectivity, accuracy, and sensitivity, and successfully applied in real-time by evaluating the levels of Cu^{2+} and CySH in a live rat brain with AD. As shown in Scheme 1, four strategies were mainly developed: (1) *N,N*-di-(2-picoly)ethylenediamine (DPEA) was synthesized as a specific recognition element for Cu^{2+} by means of coordination interactions. The electrochemical reduction peak of Cu^{2+} was obtained at 195 mV versus Ag/AgCl at the DPEA-modified electrode, and the peak current increased with the increasing concentration of Cu^{2+}. More



Scheme 1. Illustration of the single electrochemical biosensor for in vivo ratiometric monitoring of Cu^{2+} and CySH in live rat brain with Alzheimer's Disease.

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interestingly, this peak current decreased after the addition of CySH, which was attributed to the release of Cu^{2+} from the DPEA–Cu^{2+} complex when CySH bond to the Cu^{2+} center. This observation was completely different from our previous reports in which Cu^{2+} could not be released again from the complexes of TPEA and TPAA.[11a–c] (2) HS-DNA-MB with a separated peak potential at −290 mV versus Ag/AgCl was designed as an inner-reference element to provide a built-in correction for avoiding the complicated brain interferences. The peak observed at −290 mV, ascribed to MB, stayed almost constant with the changes of Cu^{2+} and CySH, resulting in the ratiometric determination of Cu^{2+} and CySH with high accuracy using a single biosensor. (3) Gold nanoleaves electrodeposited onto a carbon fiber microelectrode (CFME) improved the sensitivity toward Cu^{2+} and CySH by ca. 4.5 fold to fulfill the requirements for in vivo detection, owing to the larger surface area and higher electrocatalytic activity. (4) The significant analytical performance of this single ratiometric biosensor, as well as the unique properties of CFME, including ease of miniaturizing and good biocompatibility, established a direct and reliable approach for evaluating the in vivo levels of Cu^{2+} and CySH in a live rat brain with AD. To our knowledge, this is the first report that the concentration of Cu^{2+} increased by ca. 5-fold and that of CySH decreased by ca. 3-fold in the rat brains with AD, compared with those in the normal rat brains.

As a starting point for this work, DPEA was synthesized (Supporting Information, Figure S1), and characterized by NMR and MS (Figures S2–S4). On the other hand, Au nanoleaves were electrodeposited on the CFME surface, which was denoted as Au/CFME. From the typical SEM images (Figure 1), we can see that Au leave-like nanostructures were uniformly deposited on the CFME surface (Figure 1a and b), and the diameter of CFME is ca. 8 μm. From a close observation, it was found that the length of the Au nanoleaves is ca. 6–10 μm (Figure 1b and c). Figure 1d demonstrates energy dispersive X-ray spectroscopy (EDS) elemental mapping for a cross section of Au/CFME shown in Figure 1b. The EDS analysis indicated that the carbon center of fiber was surrounded by Au nanoleaves, which was further confirmed by XRD data (Figure S5). Then, HS-DNA-MB and mercaptopropionic acid (MPA) were modified onto CFME by a Au–S covalent bond to form MB/Au/CFME and MPA + MB/Au/CFME, respectively. Finally, DPEA was assembled onto MPA + MB/Au/CFME using 3-(3-dimethylaminopropyl)-1-ethylcarbodiimide(EDC) and N-hydroxysuccinimide (NHS). This electrode is hereafter referred to as DPEA + MB/Au/CFME. The modification processes were tracked by X-ray photoelectron spectroscopy (Figure S6).

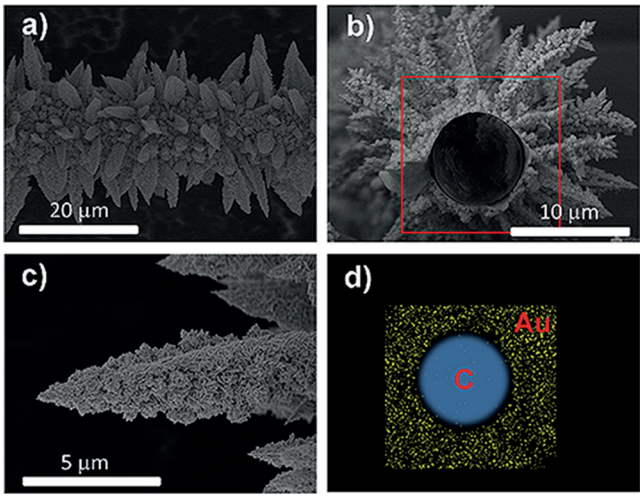

Figure 1. SEM images of Au/CFME (a), and lateral image of Au/CFME (b), the enlarged Au nanoleaves (c), and EDX analysis of selected areas of Au nanoleaves (yellow) and carbon nanofiber (blue) as shown in (d).

Considering its high sensitivity, differential pulse voltammetry (DPV) was employed for quantitative determination of Cu^{2+} and CySH under the optimized experimental conditions in artificial cerebrospinal fluid (aCSF, pH 7.4). As demonstrated in Figure 2, only a cathodic peak was clearly observed

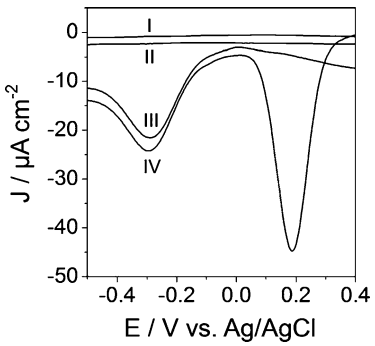

Figure 2. DPVs obtained at (I) CFME, (II) Au/CFME, (III) MPA + MB/Au/CFME in aCSF (pH 7.4), and (IV) DPEA + MB/Au/CFME in aCSF (pH 7.4) containing 10 μM Cu^{2+}.

at −290 mV versus Ag | AgCl for MPA + MB/Au/CFME (curve III), which was attributed to reduction of MB in HS-DNA-MB. No obvious responses were obtained at CFME and Au/CFME (curves I and II), even with the addition of Cu^{2+} in this potential range (data not shown). Moreover, a new cathodic peak was observed at 195 mV at DPEA + MB/Au/CFME (curve IV) in aCSF solution (pH 7.4) containing 10 μM Cu^{2+}, which was attributed to reduction of Cu^{2+} coordinated with DPEA molecules. The cathodic peak, located at 195 mV, gradually increased with increasing concentration of Cu^{2+} (Figure 3a), while that observed at −290 mV stayed almost constant, leading to the ratiometric determination of Cu^{2+} with high accuracy. The peak current density ratio (J_p/J_p^0) obtained at 195 mV and −290 mV exhibited a good linearity with the concentrations of Cu^{2+} in the range of 1–14 μM with a detection limit of 320 nM (Figure 3a, inset). The results indicated that as expected, the designed DPEA molecules showed sensitive response toward Cu^{2+} owing to the complexation of DPEA with Cu^{2+} (Figure S7). The complexation reaction time was optimized as 3 min. (Figure S8)

More interestingly, the cathodic peak obtained at 195 mV gradually decreased with increasing concentration of CySH (Figure 3b). The ratiometric peak current density (J_p/J_p^0) also plotted well with the addition of CySH from 1 μM to 12 μM

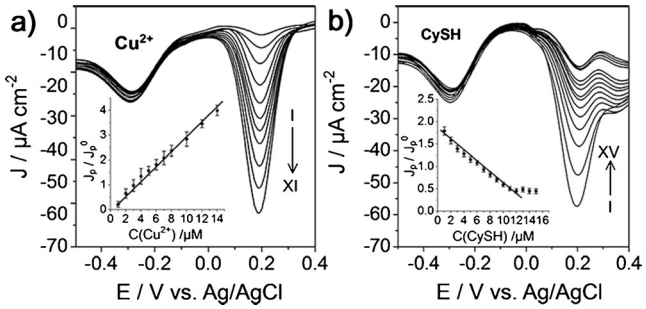

Figure 3. a) DPVs obtained at DPEA + MB/Au/CFME in aCSF (pH 7.4) with different concentrations of Cu^{2+} (From I to XI: 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14 μM). Inset: The linear relationship of the peak current density ratio obtained at 195 mV and −290 mV vs. Ag/AgCl (J_p/J_p^0) with different Cu^{2+} concentrations. b) DPVs obtained at DPEA-Cu^{2+} + MB/Au/CFME in aCSF (pH 7.4). Inset: The linear relationship of J_p/J_p^0 with different CySH concentrations (From I to XV: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 μM).

(Figure 3b, inset). The detection limit was found to be 480 nM, which fulfills the requirements for evaluating the level of CySH in a rat brain. The electrochemical signals for Cu^{2+} and CySH at DPEA + MB/Au/CFME were estimated to be ca. 4.5-fold greater than that obtained at DPEA + MB/CFME, owing to the amplified effect of Au nanoleaves (Figure S9). Thus, this single ratiometric biosensor provided a sensitive and accurate platform for determination of Cu^{2+} and CySH levels. Although we have developed several methods for detection of Cu^{2+} by our designed recognition elements, this is the first time that the single biosensor can subsequently be utilized for CySH detection after accurate detection of Cu^{2+}. This observation may be attributed to the release of Cu^{2+} from the DPEA–Cu^{2+} complex through the binding of CySH and Cu^{2+}, resulting in the precipitate (Figure S10). The observation was completely different from other complexes such as TPEA and TPAA in our previous reports.[11a–c]

The complexity of the brain system presents a significant challenge for analytical performance, not only in sensitivity and accuracy, but more importantly in selectivity. In this work, the selectivity test was evaluated by monitoring the peak current density ratio (J_p/J_p^0) induced by potential interferences, including metal ions, amino acids, ROS, and neurotransmitters that may coexist in the brain systems, against that of Cu^{2+} and CySH (Figure S11). Other metal ions showed negligible responses against determination of Cu^{2+}. Meanwhile, no obvious signals were observed for amino acid, neurotransmitters, and other potential interferences. On the other hand, the selectivity test was also carried out for CySH determination. Other amino acids demonstrated a little interference with CySH, while neurotransmitters and other potential interferences showed almost no responses. For the competition test, the effects of all these potential interferences on the electrochemical response for Cu^{2+} and CySH were investigated in detail. Relatively little changes were observed. All these results indicate high selectivity of the present single biosensor for determination of Cu^{2+} or CySH over metal ions, amino acid, neurotransmitters, and other biological molecules, which was attributed to the specific recognition of DPEA towards Cu^{2+} and the release of Cu^{2+} from DPEA–Cu^{2+} complex upon the addition of CySH.

As demonstrated above, the developed ratiometric biosensor for Cu^{2+} and subsequent CySH showed remarkable analytical performance, together with the intrinsic properties of CFME, such as easy of tissue insertion and biocompatibility, established an accurate and selective approach for evaluating the in vivo levels of Cu^{2+} and CySH in live rat brains. For in vivo experiments, the single biosensor was implanted in the left striatum, while a plastic cannula was implanted in the right striatum. Then, reference and counter electrodes were introduced in this plastic cannula. Figure 4a

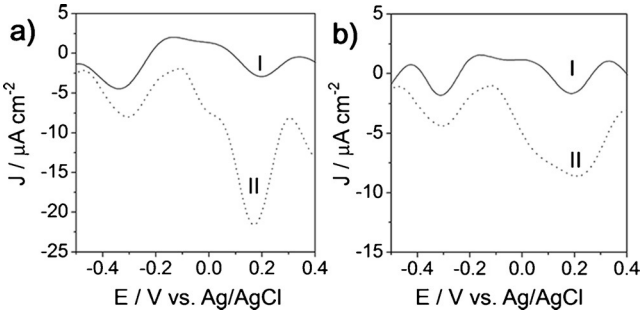

Figure 4. DPV responses obtained at a) DPEA + MB/Au/CFME for determination of Cu^{2+} and b) DPEA-Cu^{2+} + MB/Au/CFME for determination of CySH in normal rat brains (I) and rat brains with AD (II).

shows electrochemical responses for Cu^{2+} obtained at DPEA + MB/Au/CFME in the normal brain (curve I) and in the rat brain with AD (curve II). Two clear cathodic peaks were observed for the DPEA + MB/Au/CFME in the normal brain and in the rat brain with AD. It was also found that the peak current density ratio (J_p/J_p^0) increased in the rat brain with AD, compared with that in the normal brain. The concentrations of Cu^{2+} were estimated to be 1.58 ± 0.33 μM and 7.87 ± 0.08 μM for the normal brain and the rat brain with AD, respectively. Figure 4b shows electrochemical responses for CySH in the normal brain (curve I) and in the rat brain with AD (curve II). The concentrations of CySH were calculated to be 7.90 ± 0.74 μM and 2.58 ± 0.13 μM for the normal brain and the rat brain with AD, respectively. The level of Cu^{2+} increased by ca. 5-fold and that of CySH decreased by ca. 3-fold in the rat brains with AD, compared with those in the normal rat brains. Furthermore, the obtained results of Cu^{2+} and CySH in the rat brains by the developed biosensor were compared with those obtained by the off-line traditional methods (Inductively Coupled Plasma-Atomic Emission Spectrometry, ICP-AES, for Cu^{2+} and High Performance Liquid Chromatography, HPLC, for CySH), as summarized in Table 1. According to the statistical calculation by a t test ($a = 0.05$), the concentrations of Cu^{2+} and CySH in the rat brains determined by the present ratiometric biosensor were in good agreement with those obtained by the traditional methods.

In summary, by synthesizing DEPA for specific recognition element of Cu^{2+} and designing HS-DNA-MB to provide an inner-reference element, we have developed a single

Table 1: Concentrations of Cu^{2+} or CySH determined by the present method in normal rat brains and rat brains with AD, compared with those obtained by ICP-AES or HPLC from corresponding rat brain microdialysates.

Cu^{2+} [μM]	The present method				ICP-AES			
	Rat 1	Rat 2	Rat 3	Mean ± SD ($n=3$)	Rat 1	Rat 2	Rat 3	Mean ± SD ($n=3$)
normal rat brain	1.50	1.30	1.94	1.58 ± 0.33	1.57	1.23	2.03	1.61 ± 0.40
Rat brain with AD	7.94	7.79	7.87	7.87 ± 0.08	8.34	7.96	7.82	8.04 ± 0.20
CySH [μM]	The present method				HPLC			
	Rat 1	Rat 2	Rat 3	Mean ± SD ($n=3$)	Rat 1	Rat 2	Rat 3	Mean ± SD ($n=3$)
normal rat brain	7.94	7.14	8.61	7.90 ± 0.74	8.12	6.61	8.72	7.82 ± 1.09
Rat brain with AD	2.43	2.61	2.69	2.58 ± 0.13	2.84	2.90	3.05	2.93 ± 0.11

biosensor for ratiometric detection of Cu^{2+} and CySH. The present single biosensor demonstrated high selectivity against other metal ions, amino acids, ROS, and neurotransmitters, as well as high accuracy and sensitivity. The significant analytical performance, combined with the unique properties of CFME, allowed for a reliable in vivo approach for evaluating the levels of Cu^{2+} and CySH in rat brains with AD. This study has first reported the accurate concentrations of Cu^{2+} and CySH in live normal rat brains and rat brains with AD, which may be closely related to physiological and pathological events of brain. The simplicity in operation and instrumentation of this single biosensor should make it find broad applications in biochemical investigations. The present work has also provided a methodology for designing and constructing single biosensors for other metal ions, amino acids, and neurotransmitter, which play important roles in brain chemistry.

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