Biosensors

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A Two-Channel Ratiometric Electrochemical Biosensor for In Vivo Monitoring of Copper Ions in a Rat Brain Using Gold Truncated Octahedral Microcages**

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To develop in vivo monitoring strategies of physiologically important species involved in brain chemistry is a challenging work for progress in understanding the roles that biomolecules play in pathology and physiology. In brain, one of the main functions of the extracellular fluid and neurons is to convey messages as rapidly and selectively as possible. Metal ions are considered as relatively vital messengers.[1] On one hand, they are essential for many metabolic processes, and their homeostasis has been proven to be crucial to normal brain function. On the other hand, alternations in cellular metal ion concentration may cause cell death and various neurological diseases, such as Alzheimer's disease and Parkinson's disease.[2] There are many reports on that dyshomeostasis in the concentrations of, for example brain zinc, copper, and cobalt, which is considered as a risk factor for neurological disorders.[3]

Up to now, several elegant techniques have been reported for detection of Cu^{2+} ions, including atomic absorption spectrometry (AAS), inductively coupled plasma mass spectroscopy (ICP-MS), inductively coupled plasma atomic emission spectrometry (ICP-AES), fluorescence chemosensors, and electrochemical approaches.[4] Electrochemical methods have attracted more and more attention, because of the low-cost, simple instrumentation and the real-time, even in vivo detection.[5] Recently, we have reported the electrochemical detection of Cu^{2+} ions in rat brain microdialysates based on a disk electrode modified with the nanocomposites of carbon dots and *N*-(2-aminoethyl)-*N*,*N*′,*N*′-tris(pyridine-2-yl-methyl)ethane-1,2-diamine (AE-TPEA).[6] However, direct determination in complex brain is still a great challenge, because it is very hard to fulfill the requirements of analytical performance in particular sensitivity, selectivity, and accuracy, available for direct detection in the brain.

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Herein, for the first time a ratiometric electrochemical strategy is reported for the in vivo determination of Cu^{2+} ions in a rat brain (Figure 1a). Four new strategies were developed in this work. First, a Cu-free derivative of bovine erythrocyte copper–zinc superoxide dismutase (SOD—E_2Zn_2SOD; E designates an empty site) was designed and prepared for the unique biomolecular recognition for Cu^{2+} ions because E_2Zn_2SOD and Cu^{2+} ions could interact with high specificity to reconstitute SOD. The functionalized electrode showed a high selectivity for the electrochemical detection of Cu^{2+} ions against, for example other metal ions and amino acids. Second, a 6-(ferrocenyl) hexanethiol (FcHT)-modified electrode was first employed as a built-in correction electrode for avoiding environmental effects, and combined with a E_2Zn_2SOD-functionalized electrode to construct a two-channel ratiometric electrochemical biosensor, thus improving the accuracy of the biosensor for the detection of Cu^{2+} ions in the rat brain. Third, gold truncated octahedral microcages full of nanograins were synthesized for significantly improving the sensitivity because of their large surface area and high electrocatalytic activity. The sensitivity was enhanced by about seven-fold through the employment of gold microcages, and the dynamic linear range was broadened from 10 nM to 35 μM with a detection limit down to 3 nM. Finally, the significant analytical performance of the present biosensor, as well as the properties of the carbon fiber microelectrode (CFME) of a small size down to 10 μm, the easy insertion, and good biocompatibility, established a direct and reliable approach for the determination of Cu^{2+} ions in a rat brain followed by cerebral ischemia. We believe this is the first report for the direct determination of Cu^{2+} ions in a rat brain.

Typical SEM and TEM images of the as-prepared Au cages are given in Figure 2. Figure 2a indicates that the product consists purely of well-defined truncated octahedrons with a diameter distribution of about 1–1.5 μm. The size and morphology distribution of the Au microcages agrees with that of Cu_2O templates (see Figure S1 in the Supporting Information). Some crushed products in Figure 2b announce that these truncated octahedrons have big hollow interiors, which is also evident in the TEM image given in Figure 2c. A closer observation of one cage reveals that the wall of the cage is composed of nanograins with a thickness of about 100 nm on average. Abundant mesopores and splits therefore formed because of the loosely aggregated nanograins in the thick cage wall (Figure 2d). The insert in Figure 2d is the selective area electron diffraction (SAED) pattern of one gold cage, showing a ringlike pattern with many intense spots. The

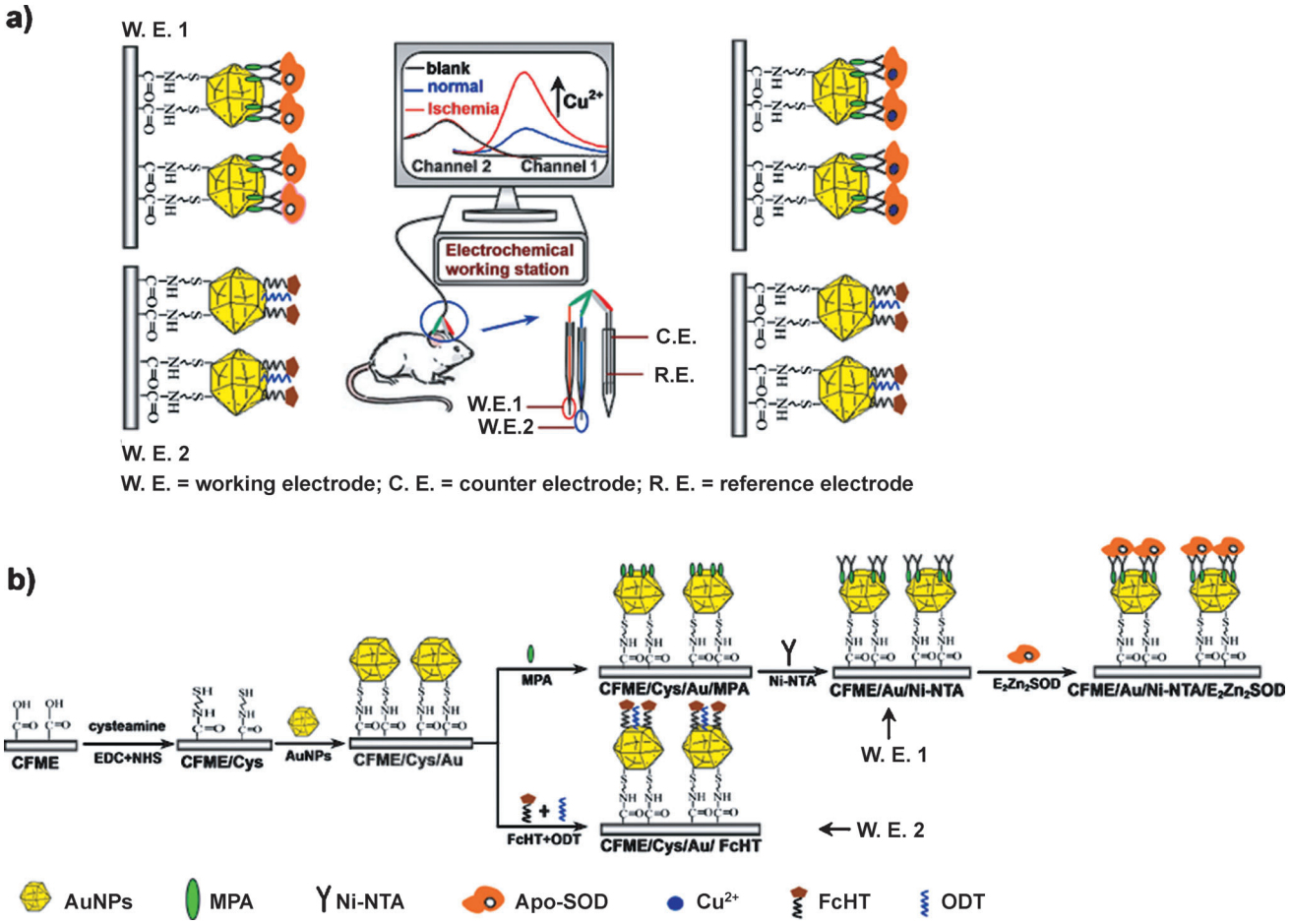

Figure 1. a) The present "turn-on" electrochemical biosensor for in vivo monitoring of cerebral Cu^{2+} in a rat brain followed by ischemia. b) Preparation procedures for the modified electrodes.

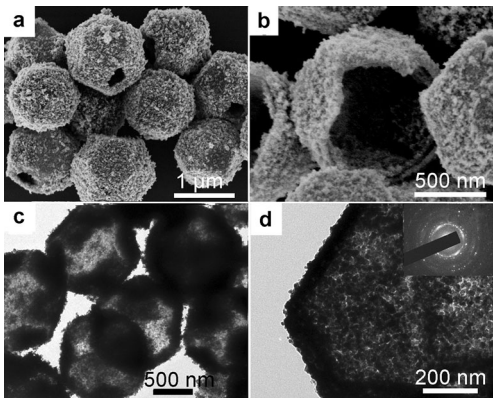

Figure 2. a,b) SEM and c,d) TEM images of the as-synthesized Au truncated octahedral cages. The inset in (d) shows an electron diffraction (ED) pattern of one cage.

ringlike pattern indicates the truncated octahedral cage is polycrystalline, and the ring signals from inside to outside could be ascribed to the (111), (200), (220), and (311) facets of a cubic phase of gold (JCPDS 04-0784). These observations were also in a good agreement with those obtained by X-ray diffraction spectroscopy (see Figure S2 in the Supporting Information).

As illustrated in Figure 1 b, the as-prepared gold octahedral microcages were attached to cysteamine (Cys) through a Au–S bond, which was assembled onto CFME in advance. The modified electrodes were denoted as CFME/Cys and CFME/Cys/Au, respectively. On one hand, for the preparation of a E_2Zn_2SOD-functionalized electrode, the gold microcages were modified with a -SH groups by 3-mercaptopropionic acid (MPA; CFME/Cys/Au/MPA) followed by the attachment of Ni-NTA (CFME/Au/Ni-NTA). The Cu-free derivative E_2Zn_2SOD was prepared according to the method described by Cocco et al.,[7] and finally conjugated with Ni-NTA because of the metal–chelate affinity.[8] The E_2Zn_2SOD-functionalized electrode was defined as CFME/Au/Ni-NTA/E_2Zn_2SOD and used hereafter.

On the other hand, for further preparation of a FcHT-functionalized electrode, CFME/Cys/Au was immersed in a mixed solution of FcHT (1 mM) and 1-octadecanethiol (ODT, 1 mM) for 12 h. The resulting electrode was denoted as CFME/Au/FcHT. The preparation procedures for the modified electrodes were tracked by X-ray photon spectroscopy (XPS; see Figures S3 and S4 in the Supporting Information) and surface plasmon resonance experiments (see Figure S5 in the Supporting Information).

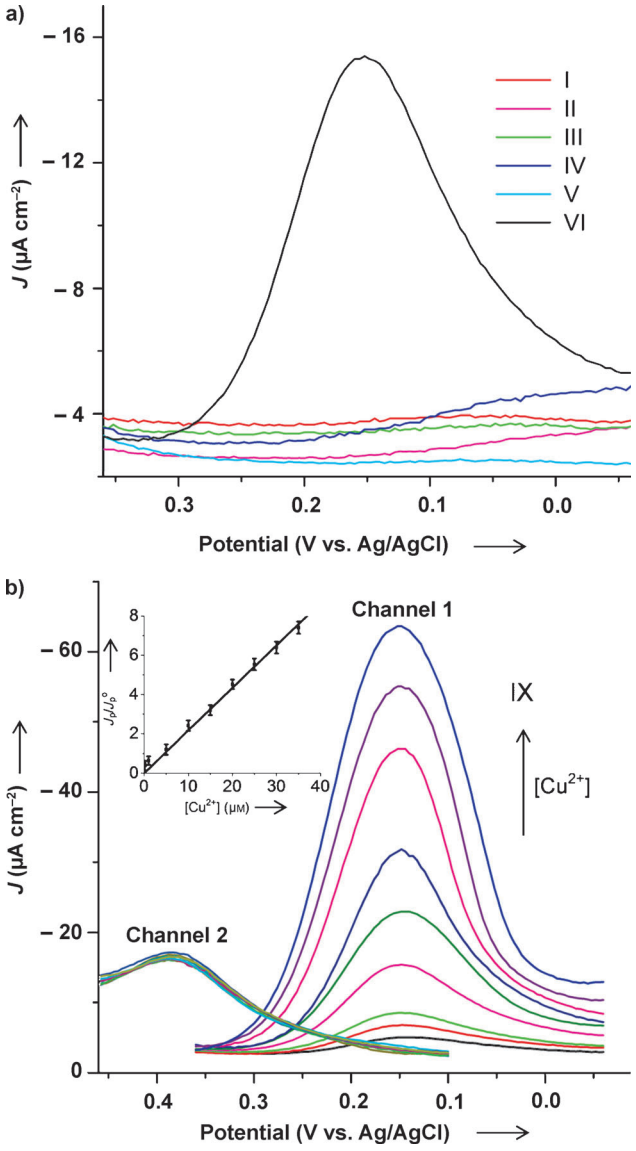

Figure 3. A) DPV responses for I) bare CFME, II) CFME/Cys, III) CFME/Cys/Au, IV) CFME/Cys/Au/MPA, V) CFME/Au/Ni-NTA, and VI) CFME/Au/Ni-NTA/E_2Zn_2SOD electrodes, respectively, in aCSF (pH 7.4) containing 5 μM Cu^{2+}. B) DPV responses of CFME/Au/Ni-NTA/E_2Zn_2SOD (channel 1) and CFME/Au/FcHT (channel 2) electrodes in aCSF (pH 7.4) containing Cu^{2+} (from I to IX) of 0.01, 0.1, 1, 5, 10, 15, 25, 30, and 35 μM. Inset: Calibration plot of the ratiometric peak current density obtained from two channels (J_p/J_p^0) versus the Cu^{2+} concentration.

Considering its high sensitivity, differential pulse voltammetry (DPV) was employed for the quantitative determination of Cu^{2+} ions. As demonstrated in Figure 3a, a cathodic peak located at 150 mV vs. Ag | AgCl was clearly observed at the CFME/Au/Ni-NTA/E_2Zn_2SOD electrode in aCSF solution (aCSF = artificial cerebral spinal fluid, pH 7.4), whereas no obvious responses were obtained at other modified electrodes. The results indicate as expected that the E_2Zn_2SOD-functionalized electrode shows good response toward Cu^{2+} ions because of the specific interaction of the designed molecule E_2Zn_2SOD with Cu^{2+} to reconstitute SOD. This observation was also evident by UV/Vis spectroscopy.[7,9] The absorption spectrum of the reconstituted SOD by Cu^{2+} ions is in a good agreement with that of native SOD with $\lambda_{max} = 680$ nm, but obviously different from that of a Cu^{2+} aqua ions and that of E_2Zn_2SOD (see Figure S6 in the Supporting Information). These results suggest that E_2Zn_2SOD can be reconstituted by simply adding Cu^{2+} ions to form SOD. In addition, the electrochemical reactions of native and reconstituted SOD were investigated by cyclic voltammetry (CV) and DPV. No voltammetric response of E_2Zn_2SOD was observed at the CFME/Au/Ni-NTA electrode in a potential range from −0.2 to 0.5 V. However, similar to that of native SOD, one pair of well-defined voltammetric peaks (CV) and a cathodic peak (DPV) appeared in the presence of the reconstituted SOD (see Figure S7 in the Supporting Information). This implies that the electron transfer of SOD is attributed to the electrochemical redox reaction of the copper moiety (not of the zinc moiety), which can be further confirmed by measuring the formal potential of the reconstituted SOD ($E^{0'}_{\text{reconstituted SOD}} = 166 \pm 13$ mV), which coincides with that of native SOD ($E^{0'}_{\text{native SOD}} = 160 \pm 14$ mV) in the error ranges as shown in Figure S8 (see the Supporting Information). Thus, the reductive peaks of native SOD and the reconstituted SOD in CVs and DPVs can be attributed to the reduction of Cu^{2+} to Cu^{+} ions (see Figures S8 and S9 in the Supporting Information). The cathodic peak density (J_p) observed at CFME/Au/Ni-NTA/E_2Zn_2SOD (channel 1) ascribed to the reduction of Cu^{2+} to Cu^{+} ions gradually increased with the increasing concentrations of Cu^{2+} ions, as depicted in Figure 3b. Under the conditions employed, the reactions became stable within 5 minutes (see Figure S10 in the Supporting Information).

On the other hand, the cathodic peak attributed to the reduction of FcHT (J_p^0) was observed at about 390 mV vs. Ag | AgCl in DPV (channel 2), as demonstrated in Figure 3b, and showed no obvious changes with increasing concentrations of Cu^{2+} ions. Thus, the CFME/Au/Ni-NTA/E_2Zn_2SOD electrode responding to Cu^{2+}, combined with the CFME/Au/FcHT electrode inert to Cu^{2+}, resulted in the two-channel ratiometric electrochemical biosensor for Cu^{2+}. The ratiometric peak currents (J_p/J_p^0) were found to be linear relative to the concentration of Cu^{2+} in the range of 10 nM to 35 μM, as shown in the inset of Figure 3. The detection limit was achieved at 3 nM, with a sensitivity of 30.2 mA M^{-1} cm^{-2}. The present strategy exhibits a wider linear range and a lower detection limit, compared with those strategies reported previously in acidic and alkali solution.[10] The sensitivity obtained at the CFME/Au/Ni-NTA/E_2Zn_2SOD electrode is about 7-fold greater than that obtained at the modified electrode without Au microcages (CFME/Ni-NTA/E_2Zn_2SOD; see Figure S11 in the Supporting Information), because of the amplified property of Au microcages possibly including the enlarged surface area and high conductance. The real surface area of CFME was enhanced by about three-fold after the modification of the Au microcages. The experimental results were well reproducible from batch to batch. The relative standard deviation (RSD) of the peak currents obtained at 10 electrodes was calculated to be 5.7%.

The complexity of the brain system presents a significant challenge for the analytical performance not only in sensi-

tivity, but more particularly in selectivity. The selectivity of the present method was evaluated by monitoring the ratiometric peak current densities (J_p/J_p^0) in aCSF solution induced by other potential interferences including, for example metal ions, amino acids, and neurotransmitters that may coexist in the brain system. No obvious responses (< 2 %) were observed upon the addition of metal ions, amino acids, and other potential interferences (see Figure S12 in the Supporting Information). For the competition test, the effect of all these potential interferences on the electrochemical response to Cu^{2+} ions was also investigated in detail in aCSF solution. Relatively little changes (< 1 %) were observed (see Figure S12 in the Supporting Information), when all the above compounds were added. These results indicate the high selectivity of the present method for Cu^{2+} relative to other metal ions, amino acids, neurotransmitters, and other biological molecules, which should be attributed to the specific recognition of E_2Zn_2SOD toward Cu^{2+}.

As demonstrated above, the developed two-channel ratiometric electrochemical biosensor for Cu^{2+} provided a reliable in vivo platform for assaying Cu^{2+} in a rat brain. For monitoring Cu^{2+} in a rat brain, the CFME/Au/Ni-NTA/E_2Zn_2SOD and CFME/Au/FcHT electrodes were implanted in the left and right striatum, respectively, while another 2 mm plastic cannula was located at about 5 mm away from one of the working electrodes. Then, reference and counter electrodes were introduced in this plastic cannula (Figure 1 a). Figure 4 shows electrochemical responses obtained at the two-channel ratiometric biosensor in the normal rat brain (curve I) and in the rat brain followed by global cerebral ischemia (curve II). A clear cathodic peak current was observed at 150 mV vs. Ag | AgCl at the CFME/Au/Ni-NTA/E_2Zn_2SOD electrode (channel 1) in the normal rat brain, whereas no current was obtained in the same potential range at pure aCSF, suggesting the presence of Cu^{2+} in the rat brain. Meanwhile, a reference peak located at 390 mV vs. Ag | AgCl was also obtained at the CFME/Au/FcHT electrode (channel 2). Furthermore, the peak current density obtained in channel 1 significantly increased in the rat brain followed by global cerebral ischemia, indicating the remarkably increased concentration of Cu^{2+}. No obvious changes in the peak current density were observed in channel 2, which was employed as a built-in correction electrode. The basal concentrations of Cu^{2+} in the normal rat brain and that followed by cerebral ischemia were estimated by the present ratiometric biosensor on average to be 2.09 ± 0.11 μM and 9.30 ± 0.13 μM, respectively, which were similar to literature values reported in the rat microdialysates.[9,11]

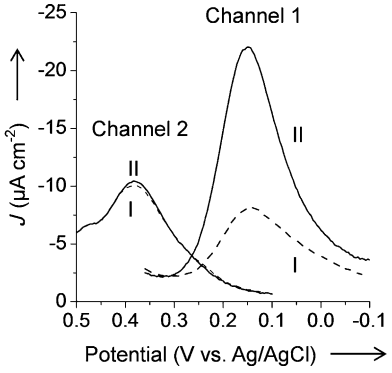

Figure 4. DPV responses obtained at the two-channel ratiometric electrochemical biosensor (channel 1: CFME/Au/Ni-NTA/E_2Zn_2SOD; channel 2: CFME/Au/FcHT) for determination of Cu^{2+} in a rat brains: I) normal rat brain and II) rat brain followed by cerebral ischemia.

The determined results of Cu^{2+} in rat brains by the developed method were compared with those obtained by the traditional method ICP-AES, as summarized in Table 1. According to the statistic calculation by a t test ($\alpha = 0.05$), the concentrations of Cu^{2+} in the rat brains determined by the present ratiometric biosensor were in good agreement with those estimated by the ICP-AES method. This comparison suggests that the developed ratiometric electrochemical method based on the combination of designed E_2Zn_2SOD and Au microcages opened up a reliable in vivo approach for determining the concentrations of Cu^{2+} in rat brain, as well as investigating the role that Cu^{2+} plays in brain chemistry.

In summary, we have first designed a Cu-free derivative of SOD, E_2Zn_2SOD, for the biomolecular recognition toward Cu^{2+} because E_2Zn_2SOD and Cu^{2+} can interact with high specificity to reconstitute SOD. Based on this molecular recognition, together with the amplified property of Au truncated octahedral microcages, we have developed a direct, selective, and sensitive strategy for the electrochemical determination of Cu^{2+} at nanomolar levels. In addtion, a FcHT-modified electrode which is employed as a built-in correction electrode for avoiding the environmental effects, has been combined with E_2Zn_2SOD-functionalized electrode to construct a ratiometric electrochemical biosensor, thus improving the accuracy of the biosensor for detection of Cu^{2+}. The present method with high sensitivity, selectivity, and accuracy has successfully been applied for the reliable detection of Cu^{2+} in a rat brain. The present work not only provides a methodology to design biosensors based on metal-free proteins for specific recognition of metal ions, but also establishes a direct and reliable approach for in vivo monitoring cerebral species in brain, which may be related to brain chemistry, and physiological and pathological events.

Table 1: Concentrations of Cu^{2+} determined by the present method in normal rat brains and rat brains followed by ischemia, compared with those obtained by ICP-AES from corresponding rat brain microdialysates (SD = standard deviation).

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	2.19	2.15	1.84	**2.09 ± 0.11**	2.39	2.17	2.05	**2.20 ± 0.14**
rat brain followed by ischemia	9.35	9.10	9.45	**9.30 ± 0.13**	9.43	9.51	9.85	**9.60 ± 0.18**

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