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A Selective and Accurate Ratiometric Electrochemical Biosensor for Monitoring of Cu^{2+} Ions in a Rat Brain

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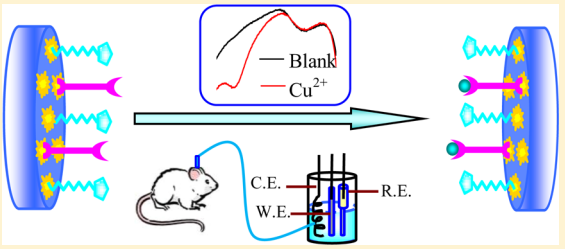
Supporting Information

ABSTRACT: Metals are essential components of all living cells, and in many cases cells trigger and utilize dynamic metal movements for signaling purposes. So, it is very critical to develop the biosensors for determination of metal ions in living systems with high selectivity and accuracy. In this work, taking Cu^{2+} as a model, an accurate and selective ratiometric electrochemical biosensor was developed. First, the specific molecule, 2,2′,2″-(2,2′,2″-nitrilotris(ethane-2,1-diyl)-tris((pyridin-2-ylmethyl)azanediyl)triethanethiol (TPAASH), was designed and synthesized for specific recognition of Cu^{2+}. Meanwhile, electroactive molecule, 6-(ferrocenyl)hexanethiol (FcHT) was coimmobilized with TPAASH at one electrode as inner reference molecule to provide a built-in correction for avoiding the environmental effects. Thus, the developed biosensor demonstrated high accuracy and remarkable selectivity toward Cu^{2+} against other metal ions, amino acids, and so on. In addition, the biosensor also showed high sensitivity due to the electrocatalytic activity of the nanostructured gold flowers. As a result, the present ratiometric electrochemical biosensor was successfully applied in detection of Cu^{2+} in brain microdialysates of normal rat brain and that followed by global cerebral ischemia.



Copper (Cu) is one of the essential components utilized in all domains of life. It is a redox-active metal that fluctuates between the oxidized (Cu^{2+}) and reduced (Cu^{+}) states. On one hand, Cu is a highly toxic metal element. The imbalance of Cu^{2+} and Cu^{+} can generate reactive oxygen species (ROS), resulting in damage events, which are connected to a variety of neurodegenerative aliments, including Menkes and Wilson's diseases, Alzheimer's disease, familial amyotrophic lateral sclerosis, and so on. On the other hand, the ability of Cu to cycle between a stable Cu^{2+} and unstable Cu^{+} is used by cuproenzymes involved in redox reactions, e.g., Cu, Zn-superoxide dismutase, cytochrome oxidase, and so on.[1−8]

Up to now, several elegant approaches have been established for determination of Cu^{2+}, including fluorescence chemosensors, atomic absorption spectrometry (AAS), inductively coupled plasma atomic emission spectrometry (ICP-AES), electrochemical strategies, and so on.[9−16] Electrochemical methods show remarkable advantages in sensitivity and simplicity, especially the real-time measurements and in vivo determination.[17−21] Our group is very interested in determination of ROS[22−26] and related biological species[27−31] including Cu^{2+} in live systems. We have designed and synthesized AE-TPEA (*N*-(2-aminoethyl)-*N*,*N*′,*N*′-tris-(pyridine-2-yl-methyl)ethane-1,2-diamine) for selective recognition of Cu^{2+} and constructed an electrochemical biosensor for determination of Cu^{2+} in rat brain microdialysates with high selectivity.[27] Recently, we have also developed a two-channel ratiometric electrochemical strategy for determination of Cu^{2+} in rat brains.[32] In that work, a Cu-free derivative of bovine erythrocyte copper−zinc superoxide dismutase (E_2Zn_2SOD) was employed as specific recognition element and immobilized on the first working electrode. Meanwhile, 6-(ferrocenyl)-hexanethiol (FcHT) was used as reference element and modified on the second electrode to provide a stable built-in correction signal, resulting in the two-channel ratiometric detection of Cu^{2+} with high specificity and accuracy. However, the two independent working electrodes would give rise to not only more complicated operation and instruments but also more serious injury for animal brains, which would not be beneficial for practical applications. More importantly, there is still some error for quantitative determination between two electrodes, since it is not easy to control the completely same modification conditions at two electrodes. Thus, it is still a challenging work to develop a more facile and accurate electrochemical biosensor for determination of Cu^{2+} in a rat brain.

In the present work, we first designed and synthesized a new recognition molecule with high selectivity toward Cu^{2+}, TPAASH. The reduction peak of Cu^{2+} on the TPAASH-functionalized electrode was observed at −0.12 V vs Ag/AgCl. Meanwhile, an inner reference element, FcHT with reduction peak at 0.32 V vs Ag/AgCl, was coimmobilized onto the same

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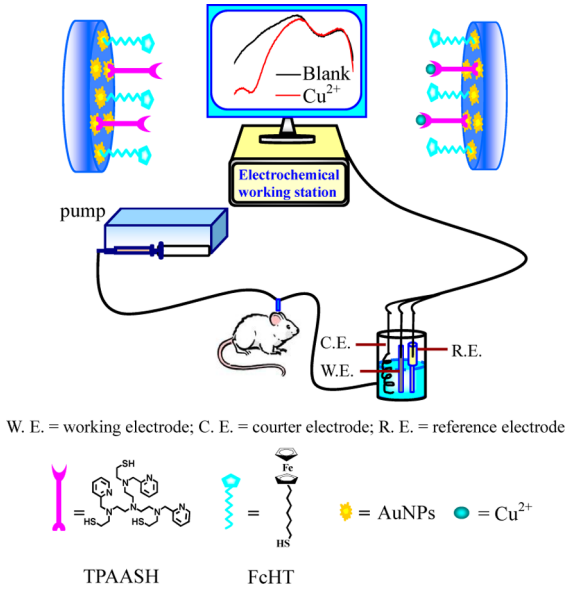
electrode together with TPAASH via one-step Au−S covalent bond, as illustrated in Scheme 1. In addition, the gold flowers

Scheme 1. Schematic Illustration of the Ratiometric Sensor for Monitoring of Cu^{2+}



were electrodeposited on the GC electrode surface for improving the sensitivity by ~2.5-fold. Finally, the new developed ratiometric electrochemical biosensor not only established a reliable approach for in vivo detection of Cu^{2+} in rat brains but also provided a more facile and accurate model for in vivo analysis of other metal ions and biological species.

EXPERIMENTAL SECTION

Reagents and Chemicals. Tris(2-aminoethyl)amine, 6-(ferrocenyl) hexanethiol (FcHT), cysteamine, uric acid (UA), ascorbic acid (AA), dopamine (DA), glucose, lactate, and all the amino acids (99%) were purchased from Sigma-Aldrich (U.S.A.) and used as received. Artificial cerebrospinal fluid (aCSF) was prepared by mixing NaCl (126 mM), KCl (2.4 mM), KH_2PO_4 (0.5 mM), $MgCl_2$ (0.85 mM), $CaCl_2$ (1.1 mM), $NaHCO_3$ (27.5 mM), and Na_2SO_4 (0.5 mM) into doubly distilled water. The solution pH was adjusted to pH 7.4. Solutions of metal ions were all prepared from their chloride salts, which are purchased from Aladdin-Reagent company (Shanghai, China). All chemicals were commercially available and of analytical grade.

Synthesis of TPAA. Tris[(2-pyridylmethyl)-2-aminoethyl]-amine pentahydrochloride monohydrate (TPAA) was synthesized as previously reported.[33] The procedure was briefly described as follows: tris(2-aminoethyl)amine (4.38 g, 30 mmol) was dissolved in ethanol (80 mL) and then dropwise added to a 100 mL ethanol solution containing 2-pyridinecarbaldehyde (9.60 g, 90 mmol) with stirring. The mixture was refluxed for 3 h under protection of N_2 gas and allowed to cool at room temperature. Next, $NaBH_4$ (1.14 g, 30 mmol) was added to the above solution, and the mixture was stirred overnight. The products were neutralized with concentrated HCl and evaporated to dryness. The residue was taken up in a saturated aqueous solution of Na_2CO_3 and extracted with dichloromethane. The organic layer was dried using $MgSO_4$ and filtered. The filtrate was evaporated to give a yellowish oil of TPAA, which was confirmed by nuclear magnetic resonance (NMR) (Supporting Information Figure S-1A). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 8.48 (dd, 3H), 7.58 (ddd, 3H), 7.31 (d, 3H), 7.12 (dd, 3H), 3.90 (s, 6H), 2.75 (t, 6H), 2.65 (t, 6H); ^{13}CNMR (125 MHz, $CDCl_3$): δ 159.79, 149.07, 136.24, 121.99, 121.69, 55.00, 54.33, 47.22 (Supporting Information Figure S-1B).

Synthesis of TPAASH. TPAA (1.257 g, 3 mmol) dissolved in trichloromethane (20 mL) was added in a small flasks with a stir bar under atmosphere of Ar gas. Ethylene sulfide (1.071 mL, 18 mmol) was added dropwise, and the mixture solution was kept at 65 °C for 4 days. Then, the solution was filtered, and the solvent was removed by rotary evaporation. Finally, the raw product was further purified by silica gel (100−200 meshes) column chromatography using methanol−ethyl acetate (1:9) as eluent. The product was a yellow oil, which was characterized by NMR and mass spectrometry (MS). ^{1}H NMR (400 MHz, $CDCl_3$) (Supporting Information Figure S-2A): δ (ppm) = 8.51 (t, 3H), 7.65 (m, 3H), 7.50 (d, 3H), 7.16 (t, 3H), 3.74 (s, 6H), 2.70−2.78 (m, 6H), 2.56−2.63 (m, 18H), 1.72 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ (ppm) = 159.67, 148.87, 136.52, 122.50, 121.81, 60.63, 59.19, 53.27, 52.58, 24.79 (Supporting Information Figure S-2B). TOF MS EI+: calculated for [M + H]+ 600.2977, found 600.2971.

Preparation and Modification of Electrodes. A glassy carbon (GC, 3.0 mm in diameter) disk electrode purchased from Bioanalytical Systems Inc. (BAS, West Lafayette, IN) was polished with 0.1 and 0.05 μm alumina slurry on a polishing cloth. Then the electrode was cleaned under bath sonication for 5 min in acetone and distilled water and thoroughly rinsed with doubly distilled water. The nanostructured gold flowers (AuFs) were electrochemically deposited on the pretreated GC electrode from aqueous solutions of 0.1 M $HClO_4$ containing 40 mM $HAuCl_4$ by applying a potential of −0.2 V vs Ag/AgCl for 2 min. The AuFs electrodeposited electrode was denoted as GC/Au. The GC/Au electrode was then soaked in an ethanol solution (N_2-saturated) of TPAASH and FcHT with optimized concentration ratio for 24 h to form a mixed monolayer at GC/Au, and the electrode was denoted as GC/Au/TPAASH +FcHT. The modified electrode was then rinsed with ethanol to remove the nonchemisorbed TPAASH and FcHT and stored at 4 °C while not in use. For comparison, the electrode only modified with TPAASH was denoted as GC/Au/TPAASH. The real electrode surface was calculated through integrating area of the cathodic peak of the cyclic voltammograms obtained with GC/Au electrodes in 0.5 M H_2SO_4 solution by the potential scan in the range −0.2 to 1.5 V vs Ag/AgCl at 100 mV s^{-1}. The surface coverages of TPAASH and FcHT were estimated to be 5.81×10^{-10} mol cm^{-2} and 0.79×10^{-10} mol cm^{-2}, respectively.

Apparatus and Measurements. Electrochemical measurements were performed on an electrochemical analyzer (CHI 660A, Shanghai, China) in deaerated aqueous solution containing 0.1 M aCSF buffer solution with a three-electrode configuration, with a GC electrode as working electrode, a Pt wire as the counter electrode, and a Ag/AgCl electrode as reference electrode (KCl-saturated, 0.197 vs normal hydrogen electrode, NHE). All measurements were carried out at ambient temperature, and the pH value was determined with a pH meter. Differential pulse voltammetry (DPV) parameters were selected as follows: scan rate of 100 mV s^{-1}, potential step of 5 mV, pulse width of 0.2 ms, pulse period of 0.5 ms, and pulse amplitude of 50 mV.

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NMR spectra were collected in $CDCl_3$ at 25 °C on a Bruker AV-400 spectrometer. All chemical shifts were recorded in the standard δ notation of parts per million. Scanning electron microscope (SEM) images were taken using a field emission gun Hitachi S-4800 scanning electron microscope operated at 1.0 kV. X-ray diffraction (XRD) was carried out by a Shimadzu XRD-6000. X-ray photoelectron spectroscopy (XPS) investigation was conducted in PHI-5000C ESCA system (PerkinElmer) with Mg Kα radiation ($h\nu$ =1253.6 eV). All binding energies (BEs) were referred to the C 1s peak (284.6 eV) arising from surface hydrocarbons (or adventitious hydrocarbon). Inductively coupled plasma atomic emission spectroscopy (ICP-AES) was conducted by a Hitachi P-4010 system.

In Vivo Microdialysis. All procedures involving animals were conducted with approval of the Animal Ethics Committee in East China Normal University, China. Surgeries for in vivo microdialysis were performed as previously reported.[34−36] Briefly, adult male Sprague−Dawley normal rats and ischemia model rats (200−250 g, Shanghai SLAC Laboratory Animal Co. Ltd., China) were anaesthetized with chloral hydrate (initial dose of 300 mg/kg, (i.p.) with additional doses of 50 mg/kg (i.p.) as needed to maintain anesthesia) and positioned onto a stereotaxic frame (Beijing Tide-Gene Biotechnology Development Centre). The microdialysis probe (CMA/110/111 Tub) was implanted in the striatum at the site of 2.5 mm anterior to bregma, 2.5 mm lateral from midline, and 7.0 mm below dura. In order to reduce injury to the rat, the microdialysis probe should be implanted into the striatum of rats slowly within 30 min with special care. Throughout the surgery, the body temperature of the animals was maintained at 37 °C with a homemade thermic blanket (Beijing Tide-Gene Biotechnology Development Centre). The microdialysis probes (CMA/110/111 Tub) were implanted into the striatum of rats and were perfused with an aCSF solution at 2 μL min^{-1} for at least 90 min for equilibration. At least 180 μL solution was finally obtained and used without further treatments. DPV was employed for measurements of Cu^{2+} in brain dialysates; for comparison, 10 μL of brain dialysates were used for ICP-AES analysis.

RESULTS AND DISUCUSSION

The typical SEM image for GC/Au is shown in Figure 1A. It is clear that AuFs were well distributed on the GC electrode like nanostructured flowers with typical size ranging from 400 to 800 nm. The crystalline orientation of AuFs was characterized by XRD pattern (Figure 1B). Three typical peaks located at 39.0°, 45.2°, 65.5° were clearly observed, corresponding to (111), (200), (220) facets, respectively. The observation indicates that the electrodeposited AuFs are composed of pure crystalline gold with the face-centered cubic structure.

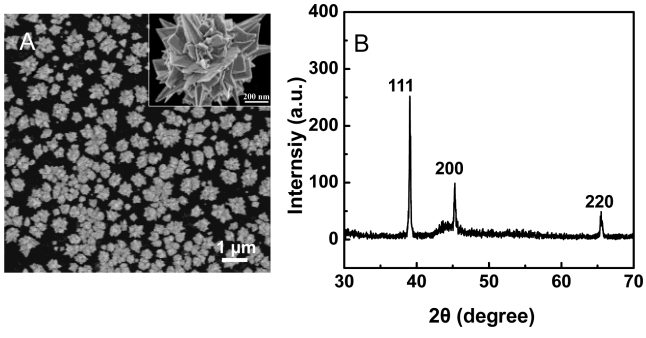

Figure 1. (A) SEM image and (B) XRD pattern of the electrodeposited Au flowers (AuFs) on GC surface; inset of A is the enlarged SEM image of AuFs.

The result was also in a good agreement with those obtained by X-ray photoelectron spectroscopy (XPS). As shown in Figure 2A, Au $4f_{7/2}$ and Au $4f_{5/2}$ peaks located at ~83.7 and 87.3 eV were clearly observed at GC/Au (curve b and c), while no peaks were obtained at bare GC substrate (curve a), indicating the successful electrodeposition of AuFs on GC substrate. The modification of TPAASH and FcHT on the GC/Au electrode was also tracked by XPS. For GC/Au/TPAASH +FcHT, obvious peaks were obtained at 164.6 eV for S 2p (curve c, Figure 2B) and at 400.8 eV for N 1s (curve c, Figure 2C), respectively. Meanwhile, two clear peaks were obtained at 710.0 and 726.0 eV for Fe $2p_{3/2}$ and Fe $2p_{1/2}$ (curve c, Figure 2D). These data suggest the successful coimmobilization of FcHT molecules with TPAASH on the GC/Au substrate via Au−S bond.

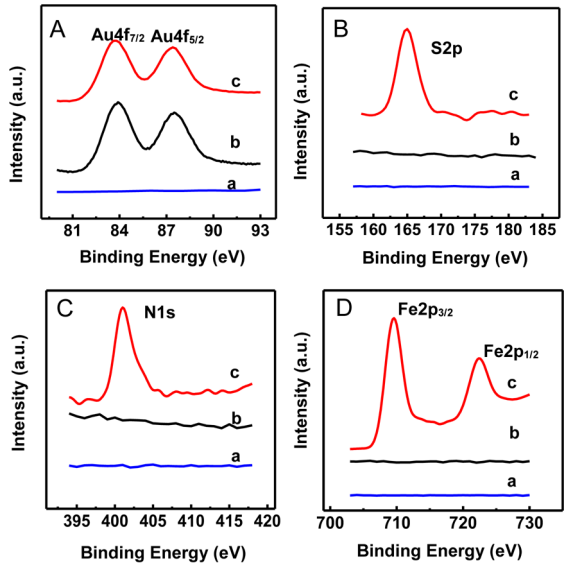

Figure 2. XPS spectra of (A) Au $4f_{7/2}$ and Au $4f_{5/2}$, (B) S 2p, (C) N 1s, and (D) Fe $2p_{3/2}$ and Fe $2p_{1/2}$ for (a) GC, (b) GC/Au, and (c) GC/Au/TPAASH+FcHT electrode.

Considering its high sensitivity, differential pulse voltammetry (DPV) was employed for the quantitative determination of Cu^{2+}. As demonstrated in Figure 3A, only one cathodic peak located at 0.32 V vs Ag/AgCl was clearly observed at GC/Au/TPAASH+FcHT in the absence of Cu^{2+} (curve c), while no obvious responses were obtained at bare GC and GC/Au electrodes in aSCF solution (curve a and b).

This cathodic peak should be ascribed to the reduction of ferrocence groups attached to the AuF-modified electrode surface. Interestingly, a new cathodic peak located at −0.12 V vs Ag/AgCl was obtained at GC/Au/TPAASH+FcHT electrode after addition of Cu^{2+}, which is attributed to the reduction reaction of Cu^{2+} recognized by TPPASH molecules at GC/Au surface. Moreover, as demonstrated in Figure 3B, this reduction peak increased with the increasing concentrations of Cu^{2+}, while the reduction peak of FcHT at 0.32 V stayed almost constant, resulting in the ratiometric determination of Cu^{2+}. The current intensity ratio between these two peaks at −0.12

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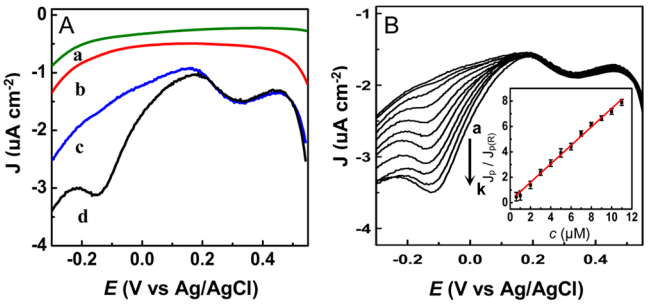

Figure 3. (A) Differential pulse voltammograms (DPVs) obtained at (a) bare GC, (b) GC/Au electrode in aCSF (pH 7.4), and GC/Au/TPAASH+FcHT electrodes in aCSF (pH 7.4) in the absence (a, b, and c) and presence of 5 μM Cu^{2+} (d). (B) DPV responses of GC/Au/TPAASH+FcHT electrode in aCSF (pH 7.4) containing Cu^{2+} (from a to k) of 0, 0.2, 1, 2, 3, 4, 5, 6, 7, 8, 9 μM. Inset: Plot of $J_p/J_{p(R)}$ obtained at GC/Au/TPAASH+FcHT electrode versus Cu^{2+} concentration.

and 0.32 V ($J_p/J_{p(R)}$, in which the peak current intensity of Cu^{2+} at −0.12 V was denoted as J_p, and $J_{p(R)}$, represents the peak current density of FcHT at 0.32 V) showed a good linearity with the concentration of Cu^{2+} ranging from 0.6 to 11.0 μM with a limit of detection (3σ), 80.3 nM (n = 3). The current density was used as response signal to compare the sensitivity between Au/TPAASH+FcHT and GC/Au/TPAASH+FcHT regardless of effect of surface area (Supporting Information Figure S-3). Thus, the sensitivity obtained at GC/Au/TPAASH +FcHT electrode is also ∼2.5 fold greater than that at the modified electrode without electrodeposited AuFs because of the amplified property of the nanostructured gold. Furthermore, the cathodic peak of FcHT exhibited negligible changes (<5.0%) during the consecutive scanning in aCSF solution for 30 min. In addition, the current density of GC/Au/TPAASH +FcHT decreases 5.4% for storing in refrigerator for 30 h in darkness, indicating this electrode had good storing stability (Supporting Information Figure S-4).

The complexity of biological sample presents a significance challenge to more particularly in selectivity. The selectivity of the present method was evaluated by monitoring $J_p/J_{p(R)}$ value in aCSF solution induced by other potential interferences including metal ions (Fe^{3+}, Zn^{2+}, Fe^{2+}, Mn^{2+}, Ni^{2+}, Co^{2+}, Pb^{2+}, Cd^{2+}, Cu^{+}, Ca^{2+}, Mg^{2+}), amino acids (Phe, Met, Gly, Glu, Cys, Arg, Lys, Leu, Ser, Thr, Val, Ile, His), and biological species (UA, AA, DA, glucose, Lact, H_2O_2, and O_2) that may coexist in the brain systems. No obvious currents were (<4%) observed for cathodic peak located at −0.12 V vs Ag/AgCl upon the addition of metal ions, amino acid, and other potential interferences (Figure 4A, C, and E). On the other hand, for the competition test, effect of all these potential interferences on the electrochemical response to Cu^{2+} was also investigated in detail in aCSF solution. Relatively little changes (<2.8%) were observed, when all the above compounds were added compared with Cu^{2+}. All the results indicate the high selectivity of the present method for Cu^{2+} over metal ions, amino acid, neurotransmitters, and other biological molecules, which is due to the specific recognition of TPAASH toward Cu^{2+}.

As demonstrated above, the detection limit of the present method is lower than that of optical sensor based on calmagite immobilized agarose membrane previously reported,[37] but the developed electrochemical method for Cu^{2+} detection showed high selectivity and accuracy, as well as long-term stability. Thus, the present biosensor was substantially applied for

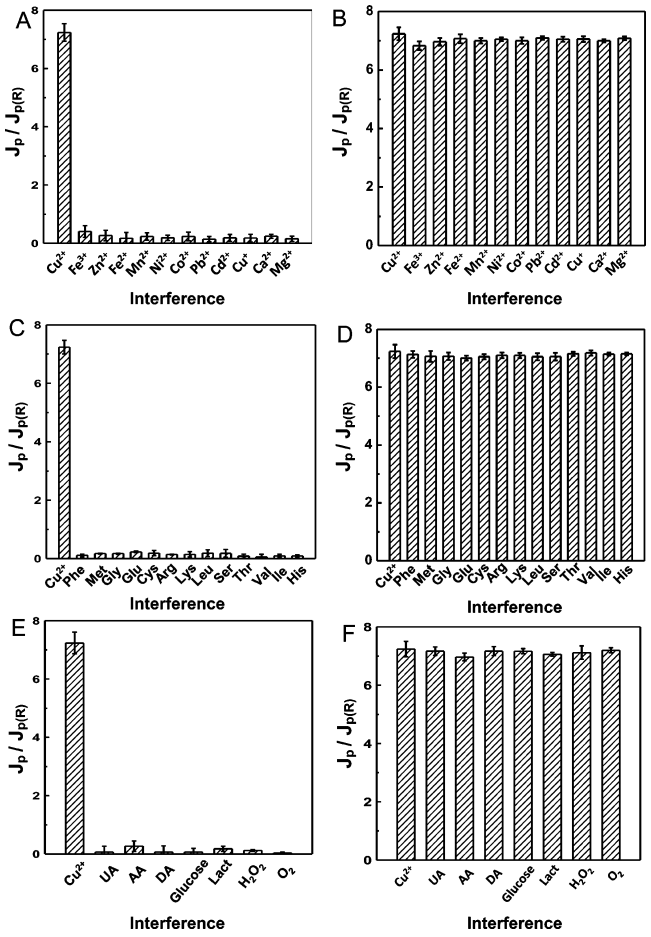

Figure 4. Selectivity (A, C, and E) and competition (B, D, and F) tests for Cu^{2+} detection in the presence of different physiological species. (A) Selectivity of metal ions against Cu^{2+}. Concentration of metal ions is 10 μM, expect concentration of Ca^{2+}, Mg^{2+} is 1 mM. (B) Competition experiments with the subsequent addition of 10 μM Cu^{2+} to the solutions containing other metal ions. (C) Selectivity of amino acids against Cu^{2+}: phenylalanine (Phe), methionine (Met), glycine (Gly), glutamic acid (Glu), cystine (Cys), arginine (Arg), lysine (Lys), leucine (Leu), serine (Ser), threonine (Thr), valine (Val), isoleucine (Ile), and histidine (His). Concentration: 10 μM. (D) Competition test with the subsequent addition of 10 μM Cu^{2+} to the solution containing amino acids. (E) Selectivity of biological species against Cu^{2+}: UA (10 μM), AA (10 μM), DA (10 μM), glucose (1 mM), lactate (1 mM), H_2O_2 (0.5 μM), and O_2 (0.2 mM). (F) Competition experiments with the subsequent addition of 10 μM Cu^{2+} to the solution containing biological species.

assaying of Cu^{2+} in rat brains, combined with in vivo microdialysis. As shown in Figure 5a, a clear peak was obtained at −0.12 V vs Ag/AgCl at GC/Au/TPAASH+FcHT electrode in normal brain microdialysates, although no response at this potential was observed in pure aCSF. The basal level of Cu^{2+} in the brain dialysates was estimated to be 2.01 ± 0.32 μM, which is similar to the reported values.[5,6] Furthermore, the peak current remarkably increased in the rat brain microdialysates followed by global cerebral ischemia, and the concentration of Cu^{2+} was estimated as 9.03 ± 1.70 μM in the rat brain after global cerebral ischemia, which is also in good agreement with the literature value.[27,32,38] These results demonstrate the significantly increasing concentration of Cu^{2+} in the rat brain after global cerebral ischemia. The traditional ICP-AES method for determination metal ions was used as a standard method to

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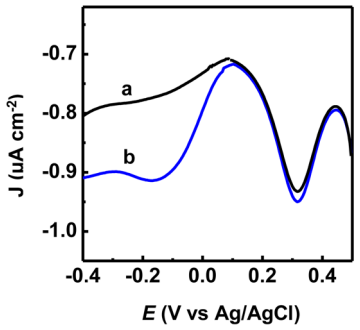

Figure 5. DPV responses obtained at GC/Au/TPAASH+FcHT electrode for the microdialysates collected from the striatum of (a) normal rat brain and (b) rat brain followed by cerebral ischemia.

evaluate the accuracy of ratiometric electrochemical sensor. The concentrations of Cu^{2+} in rat brain microdialysates determined by the present method were compared with those obtained by the traditional method ICP-AES. According to the statistic calculation by a t test (a = 0.1) (calculation of t value was shown in the Supporting Information), the concentrations of electrochemical sensor coincided well with those monitored by ICP-AES method (t = 0.19 for normal rat, t = 0.07 for rat brain followed by ischemia, which are smaller than the standard t value of 2.13), suggesting the determination results of the present method are consistent with those of ICP-AES. For further confirming the accuracy of the present ratiometric sensor for Cu^{2+}, the normal "turn-on" biosensor, GC/Au/TPAASH, was used to determine Cu^{2+} in the same brain microdialysates, and the results were also quantitatively compared with the ICP-AES method shown in Table 1. The calibration curve obtained at GC/Au/TPAASH (Supporting Information Figure S-5) in aCSF was employed to determine the concentrations of Cu^{2+} in the brain microdialysates. As summarized in Supporting Information Table S1, t was estimated to be 2.89 and 2.85 for rat brain microdialysates collected under normal and ischemia models, respectively (Supporting Information Table S-1), which are both greater than standard 2.13 value. This comparison suggests that the developed ratiometric electrochemical method by using FcHT as a built-in correction is more accurate than the conventional electrochemical turn-on biosensor without inner reference. Thus, we believe that the ratiometric electrochemical biosensor should provide a more accurate and reliable model for determination of Cu^{2+} and other metal ions in rat brains and other complicated biological systems.

CONCLUSIONS

In summary, an accurate and selective ratiometric electrochemical biosensor has been developed for determination of Cu^{2+} through one-step coimmobilization of the specific recognition and inner reference molecules on the nanostructured surface. The present biosensor with theoretically simple, and less instrumental demands, has demonstrated high selectivity, sensitivity, and accuracy. Thus, the electrochemical strategy has been successfully used for the reliable detection of Cu^{2+} in rat brain microdialysates. The simplicity in operation and miniaturation, together with the remarkable analytical performance of the biosensor, should make it very useful and powerful for further applications in biochemical investigations.

This investigation has also provided a methodology for designing and constructing ratiometric electrochemical biosensors with high selectivity and accuracy for monitoring of metal ions and other cerebral species, which may be related to the physiological and pathological processed in brain chemistry.

ASSOCIATED CONTENT

Supporting Information

^{1}H NMR and ^{13}C NMR spectra; sensitivity of Au/TPAASH +FcHT and GC/Au/TPAASH+FcHT electrodes; stability of GC/Au/TPAASH+FcHT electrodes; DPV response of GC/Au/TPAASH electrode and linearity curve; determination of Cu^{2+} using GC/Au/TPAASH electrode and t test. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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Table 1. Determination of Cu^{2+} in the Brain Microdialysates by the Present Method and by ICP-AES

	present method				ICP-AES			
Cu^{2+} (μM)	rat 1	rat 2	rat 3	mean ± SD (n = 3)	rat 1	rat 2	rat 3	mean ± SD (n = 3)
samples from normal rat brain	2.34	1.99	1.71	2.01 ± 0.32	2.15	1.83	1.92	1.97 ± 0.17
samples from rat brain followed by ischemia	10.2	7.79	9.10	9.03 ± 1.70	9.87	8.23	9.22	9.11 ± 0.82

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