Highly Selective Electrochemical Strategy for Monitoring of Cerebral Cu\textsuperscript{2+} Based on a Carbon Dot-TPEA Hybridized Surface

Xiangling Shao,\textsuperscript{†} Hui Gu,\textsuperscript{‡} Zhen Wang,\textsuperscript{†} Xiaolan Chai,\textsuperscript{†} Yang Tian,\textsuperscript{*}‡ and Guoyue Shi\textsuperscript{*}‡

\textsuperscript{†}Department of Chemistry, Tongji University, Siping Road 1239, Shanghai 200092, People’s Republic of China, and
\textsuperscript{‡}Department of Chemistry, East China Normal University, Dongchuan Road 500, Shanghai 200241, People’s Republic of China

Supporting Information

**ABSTRACT:** Direct determination of cerebral metal ions in small volume biological samples is still the bottleneck for evaluating the roles that metal ions play in the physiological and pathological processes. In this work, selected copper ion (Cu\textsuperscript{2+}) as a model, a facile and direct electrochemical method for detection of Cu\textsuperscript{2+} has been developed on the basis of two new designed strategies: one is specific recognition molecule for Cu\textsuperscript{2+}-AE-TPEA (N-(2-aminoethyl)-N,N',N'-tris(pyridine-2-yl-methyl)ethane-1,2-diamine); another is carbon dots (C-Dots) with high electrocatalytic activity. Based on the high affinity between TPEA and Cu\textsuperscript{2+}, the electrode assembled with C-Dot-TPEA hybridized nanocomposites shows high selectivity toward Cu\textsuperscript{2+} over other metal ions, amino acids, and biological coexisting species, such as uric acid (UA), ascorbic acid (AA), and so on, which makes it possible to be used for determination of Cu\textsuperscript{2+} in the complex brain system. By taking advantage of C-Dots, a dynamic linear range from 1 \(\mu\)M to 60 \(\mu\)M is first achieved with a detection limit of \(\sim 100\) nM in aCSF solution. In addition, the developed method with theoretical simplicity and less instrumental demands exhibits long-term stability and good reproducibility. As a result, the present strategy has been successfully applied in detection of cerebral Cu\textsuperscript{2+} in normal rat brain and that followed by global cerebral ischemia, combined with in vivo microdialysis. The determined concentrations of Cu\textsuperscript{2+} in the rat brain microdialysates by the present method are found to be identical to those obtained by the conventional ICP-AES method.

Copper is an essential trace element which ranks third in abundance in the human body, and copper ion (Cu\textsuperscript{2+}) plays an essential role in many metabolic processes.\textsuperscript{1} As a catalytic cofactor for a variety of enzymes, such as cytochrome c oxidase, superoxide dismutase, and tyrosinase, alterations in the catalytic cofactor for a variety of enzymes, such as cytochrome c oxidase, superoxide dismutase, and tyrosinase, alterations in the concentration of brain copper has been considered as one of the risk factors for neurological disorders.\textsuperscript{2} Thus, determination of cerebral Cu\textsuperscript{2+} has become a hot topic in the biological and analytical sciences. So far, several elegant techniques have been developed for detection of Cu\textsuperscript{2+}, including atomic absorption spectrometry (AAS),\textsuperscript{3} inductively coupled plasma mass spectroscopy (ICP-MS),\textsuperscript{4} inductively coupled plasma-atomic emission spectroscopy (ICP-AES),\textsuperscript{5} fluorescence chemosensors,\textsuperscript{6} and electrochemical approaches.\textsuperscript{7,8} Electrochemical methods have attracted more attention, because of the low-cost, simple instrumentation, and easy to real time and in situ detection.\textsuperscript{9} Despite continuing progress in improving the detectability of analytical methods, the direct determination of cerebral Cu\textsuperscript{2+} in small volume biological samples is still the bottleneck for understanding the role that Cu\textsuperscript{2+} plays in the biological and physiological systems, because it is a challenge to enhance the analytical performance especially selectivity and sensitivity, available for the applications in a complex brain system.

Herein, we developed a selective and sensitive electrochemical approach for direct determination of cerebral Cu\textsuperscript{2+} in rat brain microdialysates based on two novel strategies, as illustrated in Scheme 1. On one hand, a specific recognition molecule – AE-TPEA (N-(2-aminoethyl)-N,N',N'-tris-(pyridine-2-yl-methyl)ethane-1,2-diamine) was designed for improving the selectivity against other metal ions, because the functional molecule could coordinate with Cu\textsuperscript{2+} with high specificity to form a stable complex. On the other hand, carbon dots (C-Dots) were employed for enhancing the sensitivity of electrochemical detection, since they show unique electrochemical properties, such as strong ability for electron transfer, high catalysis activity, and large contact area due to the decreasing particle size.\textsuperscript{7a,b,11,12} The C-Dots were attached on (3-aminopropyl)trimethoxysilane (APTMS) with a methoxy functional group through silanization interaction,\textsuperscript{13} which was assembled through electrochemical scanning to form a carbon—nitrogen linkage at the glassy carbon (GC) surface in advance. Then, the receptor AE-TPEA was conjugated to C-Dots functionalized with –COOH and –OH groups through EDC and NHS as catalysts. The developed electrochemical biosensor

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showed high selectivity for Cu\(^{2+}\) over other metal ions, amino acids, and biological coexisting species, because of specific recognition of the TPEA molecule. Meanwhile, a linear range from 1 \(\mu\)M to 60 \(\mu\)M was obtained, and detection limit was achieved to \(\sim 100\) nM (based on a signal-to-noise ratio of \(S/N = 3\)). Accordingly, the designed electrode conjugated with C-Dots and AE-TPEA was successfully applied to determine the concentrations of Cu\(^{2+}\) in rat brain microdialysates. To the best of our knowledge, it is the first report that a facile electrochemical strategy was developed for determination of cerebral Cu\(^{2+}\) in a rat brain.

**EXPERIMENTAL SECTION**

**Reagents and Chemicals.** Hydrazine hydrate (\(\text{N}_2\text{H}_4\cdot\text{H}_2\text{O}\)), (3-aminopropyl)trimethoxysilane (APTMS), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), N-hydroxysuccinimide (NHS), N-(2-bromoethyl)phthalimide, and all the amino acids (99%) were purchased from Sigma-Aldrich (USA) and used as received. Artificial cerebrospinal fluid (aCSF) was prepared by mixing NaCl (126 mM), KCl (2.4 mM), KH\(_2\)PO\(_4\) (0.5 mM), MgCl\(_2\) (0.85 mM), NaHCO\(_3\) (27.5 mM), Na\(_2\)SO\(_4\) (0.5 mM), and CaCl\(_2\) (1.1 mM) into doubly distilled water, and the solution pH was adjusted pH 7.4. Solution of Ag\(^+\) was prepared from its nitride salt, and all the other solutions of metal ions were all prepared from their chloride salts with doubly distilled water. All chemicals were commercially available and of analytical grade.

**Preparation of C-Dots.** C-Dots were prepared as previously reported through the electrochemical method.\(^7a,b,14\) In brief, 0.2–0.4 g of NaOH was dissolved in 100 mL of ethanol/H\(_2\)O (99.5:0.5, v:v). By using graphite rods (diameter 0.5 cm) as both anode and cathode, C-Dots were obtained in the electrolyte with a current intensity 20 mA cm\(^{-2}\) for 10 h. The crude product of C-Dots was treated by adding an appropriate amount of MgSO\(_4\) (5–7 wt %), then stirred for 1 h, and finally stored for 24 h to remove the waters and salts. Afterward, the purified C-Dots solution was separated by silica-gel chromatography with a mixture of dichloromethane and ethanol as the developing solvent.

**Synthesis of AE-TPEA.** AE-TPEA was prepared using a modified literature procedure.\(^15\) Anhydrous K\(_2\)CO\(_3\) (3.10 g, 22.5 mmol) and KI (0.166 g, 1 mmol) were added to 100 mL of CH\(_3\)CN, followed by 3.33 g (10 mmol) of TPEA and 2.53 g (10 mmol) of N-(2-bromoethyl)phthalimide. The mixture was allowed to reflux overnight under a nitrogen atmosphere. After having been cool, it was filtered and concentrated under vacuum to yield a red oil. This red oil was dissolved in CH\(_2\)Cl\(_2\) (100 mL) and was washed with saturated NaHCO\(_3\) solution (3 \(\times\) 100 mL) and with water (2 \(\times\) 100 mL). After the solvent was evaporated in vacuo, 2 M HCl (30 mL) was added to the resulting dark oil. The aqueous solution was washed with CH\(_2\)Cl\(_2\) (6 \(\times\) 60 mL), and then it was basified with solid...
solution was added, and the mixture was stirred for another hour. Then the solution was cooled, 8.33 mL of 12 M HCl (100 mmol) was extracted three times with CH$_2$Cl$_2$. The combined CH$_2$Cl$_2$ was added to this aqueous mixture. The aqueous phase was concentrated under vacuum. 15% aqueous NaOH (150 mL) was added to this aqueous mixture. The aqueous phase was extracted three times with CH$_2$Cl$_2$. The combined CH$_2$Cl$_2$ extracts were dried over Na$_2$SO$_4$, filtered, and then concentrated under vacuum. The product was obtained as a brown oil (1.60 g, 42.8%). $^1$H NMR (400 MHz, CDCl$_3$): 8.53–8.48 (m, 3H, C$_6$H$_4$N), 7.64 (s, 2H, CH$_2$), 7.27 (m, 6H, CH$_2$CH$_2$), 2.51 (t, 2H, CH$_2$CH$_2$J $= 5.9$ Hz). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 159.7, 159.4, 148.7, 148.6, 136.1, 136.0, 122.6, 122.5, 121.7, 121.6, 60.8, 60.5, 57.5, 52.3, 52.1, 39.5. TOF MS EI$: calculated for [M+H]$^+$ *376.2375*, found *376.2377*.

**Preparation and Modification of Electrodes.** Glassy carbon (GC, 3 mm in diameter) (Chenhu, Shanghai) electrodes were first polished with emery paper, 1 and 0.05 $\mu$m alumina slurry on a polishing cloth, then cleaned under bath sonication for 10 min in distilled water, and finally thoroughly rinsed with doubly distilled water. Next, the GC electrode were scanned from −1 V to +1 V and back at a scan rate of 200 mV s$^{-1}$ for 100 cycles in a solution of 0.5 M H$_2$SO$_4$. Then, the GC electrode was immersed into a 5% (v/v) solution of (3-aminopropyl)trimethoxysilane (APTMS) in ethanol and cyclic within a potential range from 0.5 to 1.4 V at a scan rate of 10 mV s$^{-1}$ to form a carbon–nitrogen linkage at the GC electrode surface, resulting in the assembly of APTMS (step a, Scheme 1B). The resulting electrode functionalized with a methoxy functional group on the surface was referred to as the GC/APTMS electrode. Furthermore, the GC/APTMS electrode was immersed into an ethanol solution containing C-Dots for 24 h in a thermostatted water bath at 28°C. After being washed with distilled water and dried with nitrogen gas, the electrode was treated at 40°C using a drying oven for 1 h (step b, Scheme 1B). Thus, the electrode modified with C-Dots functionalized with a carboxyl group was referred to as GC/ Mar/AIME/CD.

Finally, AE-TPEA was assembled on the electrode surface by using EDC and NHS as catalysts to form the amide between the group of −COOH at C-Dots and the −NH$_2$ moiety at the AE-TPEA. The GC/ AIME/CD electrode was immersed into the AE-TPEA (2 mM) solution of ethanol containing the EDC- NHS mixture (20 mM) and stirred for 3 h for the conjugation (step c, Scheme 1B). After being rinsed with distilled water, the modified GC electrode was dried at ambient temperature and denoted to GC/AIME/CD/TPEA. Before electrochemical experiments, the modified electrode was cycled within a potential range from −0.4 to +0.2 V at a scan rate of 100 mV s$^{-1}$ until a stable cyclic voltammogram was obtained (typically after 50 cycles). The TPEA-modified electrode (GC/TPEA) was obtained by conjugation of AE-TPEA with the GC electrode functionalized with the −COOH group through EDC and NHS.

**Apparatus and Measurements.** Electrochemical experiments were performed with a CHI 832 electrochemical workstation (Chenhu, Shanghai) with a conventional three-electrode cell in 0.01 M phosphate buffer (pH 7.4). The bare or modified GC electrodes were used as working electrodes, and a Pt coil was the counter electrode. All potentials used were biased versus the Ag/AgCl electrode (saturated with KCl). All measurements were carried out at ambient temperature, and the pH value was calibrated with a pH meter. DPASV parameters were as follows: deposition time of 240 s, deposition potential of −0.4 V, scan rate of 100 mV s$^{-1}$, scan range of −0.3 to −0.2 V, potential step of 5 mV, pulse width of 25 ms, pulse period of 100 ms, and pulse amplitude of 50 mV. Nuclear magnetic resonance (NMR) spectra were collected in CDCl$_3$ at 25°C on a Bruker AV-400 spectrometer. All chemical shifts were recorded in the standard $\delta$ notation of parts per million. Electrospray mass spectrometry (ES-MS) and high-resolution mass spectral analysis were carried out on GCT Premier Time FOR flight mass spectrometer (USA) at Shanghai Key Laboratory of Green Chemistry and Chemical Processes. Fourier transform infrared spectroscopy (FT-IR) was recorded on a Nicolet model 6700 Fourier-transform infrared spectrometer. An X-ray photoelectron spectroscopy (XPS) investigation was conducted in a PHI-5000 ESCA system (Perkin-Elmer) with Mg K$_\alpha$ radiation ($h\nu = 1253.6$ eV). All binding energies (BEs) were referred to as the C 1s peak (284.6 eV) arising from surface hydrocarbons (or adventitious hydrocarbon). Atomic force microscope (AFM) images were recorded by Picoscan 2100 MI, USA. Inductively coupled plasma atomic emission spectroscopy (ICP-AES) was conducted a Hitachi P-4010 system (Japan).

**In Vivo Microdialysis.** All procedures involving animals were conducted with approval of the Animal Ethics Committee in East China Normal University (ECNU), China. Surgeries for in vivo microdialysis were performed as reported previously. Briefly, adult male Sprague–Dawley normal rats and ischemia model rats (200–250 g, Shanghai SLAC Laboratory animal Co. Ltd., China) were anesthetized 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 Center). 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 homeothermic blanket (Beijing Tide-Gene Biotechnology Development Center). The microdialysis probes (CMA/110/111 Tub) were implanted into the striatum of rats and were perfused with a CSF solution at 2 $\mu$L min$^{-1}$ for at least 90 min for equilibration. Differential pulse anodic stripping voltammetry (DPASV) was employed for measurements of Cu$^{2+}$ in rat brain. After the electrochemical experiments, 5 $\mu$L of brain dialysates were collected for ICP-AES analysis from an anesthetized rat brain striatum.

**RESULTS AND DISCUSSION.**

**Characterization of the Modified GC Electrode.** The process for the modification of electrodes illustrated in Scheme 1 was described as above in the Experimental Section. First of all, FT-IR spectroscopy was employed to characterize the
surface of bare GC surface after being treated in acidic solution. As shown in Figure 1a, the band observed at ~1638 cm$^{-1}$ is attributed to carbonyl (C=O) groups, while the band located at ~3500 cm$^{-1}$ suggests the existence of −OH. These data indicated the bare GC surface was covered with −COOH and −OH groups. Then, APTMS was assembled on the GC electrode via carbon–nitrogen linkage formed by electrochemical scanning (Step a in Scheme 1). From Figure 2b, the band obtained at 1583 cm$^{-1}$ is assigned to −CONH, and the bands ~2862 and 2930 cm$^{-1}$ are ascribed to C–H stretching. Furthermore, the bands located at ~1032 and 1128 cm$^{-1}$ are attributed to Si–O–C stretching, while the band ~932 cm$^{-1}$ results from Si–O vibration. These characteristic peaks confirmed that APTMS was successfully linked to the GC electrode surface. The modification of APTMS was also verified by X-ray spectroscopy (XPS). As demonstrated in Figure 2A (curve b), after the modification of APTMS, an obvious peak located at 102.8 eV for Si$_{2p}$ was observed, suggesting the existence of methylsiloxane on the GC surface. Meanwhile, a peak was also obtained at 399.8 eV for N$_{1s}$ (curve b, Figure 2B), which was assigned to the amine nitrogen of APTMS. For the control experiment, no clear peaks were observed for Si$_{2p}$ and N$_{1s}$ (curve a, Figure 2A and B) on the bare GC surface.

Next, C-Dots functionalized with −COOH and −OH groups were conjugated with methoxy functional group of APTMS, as illustrated in Scheme 1 (Step b). As shown in Figure 2C, the bands at around 2973 and 1593 cm$^{-1}$ are attributed to the C−C stretching modes of polycyclic aromatic hydrocarbons, while the peak located at 1664 cm$^{-1}$ shows the existence of carbonyl (C=O) groups. The peaks observed at ~1723 and ~1095 cm$^{-1}$ are assigned to the −COOH group, and that located at ~3422 cm$^{-1}$ is attributed to the OH stretching mode. These data are identical with those for pure C-Dots (Figures S5, Supporting Information). The results demonstrated that the C-Dots were successfully conjugated onto the GC surface and were still surrounded by −COOH and −OH groups, which are very beneficial for further modification of AE-TPEA. The assembly of C-Dots on the GC surface was also directly confirmed by the AFM image. From Figure 2C, the AFM image shows that the as-prepared C-Dots are monodispersed with an average size of ~6 nm, and the average thickness of C-Dots is ~2 nm.

As demonstrated in Figure 2D, C-Dots were clearly assembled on the GC surface without obvious changes in size or shape.

The structure of synthesized AE-TPEA was confirmed by $^1$H NMR, $^{13}$C NMR, ES-MS, HR-MS, and IR spectra (Figures S1–S4, Supporting Information). The specific molecule AE-TPEA with the −NH$_2$ group was covalently linked with C-Dots functionalized with the −COOH group through EDC and NHS as catalysts (Step c, Scheme 1). As shown in Figure 1c, the band at ~3390 cm$^{-1}$ is assigned to the stretching vibration of N−H groups, while the peaks located at ~1474 cm$^{-1}$ and 1673 cm$^{-1}$ are attributed to the bending vibration of the N−H group and the stretching vibration of C=O groups, respectively. These results indicated the successful modification of AE-TPEA on the C-Dots surface, which was also confirmed by XPS data. As demonstrated in Figure 2A and B, no obvious change was observed for Si$_{2p}$ spectrum (curve d, Figure 1A). However, the peak position of N$_{1s}$ shifted a little to a lower binding energy value of 399.2 eV (curve d, Figure 1B) with increased intensity after the modification of AE-TPEA, possibly due to the pyridine ring nitrogen of AE-TPEA.

**Electrochemical Responses of the CQDs/AE-TPEA Electrode.** Differential pulse anodic stripping voltammetry (DPASV) was employed for electrochemical measurements of Cu$^{2+}$ because of its high sensitivity. Figure 3A shows DPASV signals obtained at (a) bare GC, (b) GC/APTMS, (c) GC/APTMS/CD, and (d) GC/APTMS/CD/TPEA electrodes in 10 mM PBS (pH 7.4) containing 5 μM Cu$^{2+}$. A clear anodic peak located at ~50 mV vs Ag/AgCl was observed at the GC/APTMS/CD/TPEA electrode in 10 mM PBS (pH 7.4).
containing 5 μM Cu²⁺, while no obvious responses were obtained at other modified electrodes. The results indicate that as expected that the GC/APTMS/CD/TPEA electrode shows good response toward Cu²⁺ due to the effective combination of designed molecule AE-TPEA with Cu²⁺ through coordination interaction. The stability constants of the Cu(II)-TPEA complex was previously reported to be log \( K_{Cu(II)L} \) = 9.35.21 Thus, the developed GC/APTMS/CD/TPEA electrode provides a strong basis to construct a sensor for detection of cerebral Cu²⁺.

Figure 3B depicted the DPASV responses at the GC/APTMS/CD/TPEA electrode in aCSF (pH 7.4) containing different concentrations of Cu²⁺. The peak current at −50 mV vs Ag/AgCl clearly increased with the increasing concentration of Cu²⁺, and the relationship between peak current and concentration of Cu²⁺ was plotted in the inset of Figure 3B. A linear range was obtained from 1 to 60 μM with a linear correlation of 0.995, and the detection limit was calculated to be ∼100 nM (based on a signal-to-noise ratio of S/N = 3). The detection limit is not higher than those previously reported by electrochemical approaches in acidic or alkali solutions, but it is the first report that the modified electrode can be used in electrochemical determination of Cu²⁺ in the neutral aCSF solution. Moreover, the analytical performance of the modified electrode improved much better after modification of C-Dots. The sensitivity increased by 10-fold at the GC/APTMS/CD/TPEA electrode, compared with that obtained at the GC/TPEA electrode. The detection limit was lowered from 3 μM to 100 nM (Figure S7, Supporting Information).

**Selectivity, Stability, and Reproducibility.** The complexity of the brain system presents a great challenge to the analytical methods for metal ions detection not only in sensitivity but also more importantly in selectivity. Then, the selectivity experiments were carried out by monitoring the peak current in the potential range of −0.3 to 0.2 V vs Ag/AgCl of the GC/APTMS/CD/TPEA electrode in aCSF solution with the addition of other metal ions that may coexist in the rat brain. First, various metal ions such as abundant cellular cations (Na⁺, K⁺, Ca²⁺, Mg²⁺) with 100-fold concentration as that of Cu²⁺, trace metal cations in organisms (Fe²⁺, Zn²⁺, Ni²⁺, Pb²⁺, Cd²⁺, Mn²⁺, Co²⁺, and Fe³⁺) with the same concentration as that of Cu²⁺, and so on were tested. Remarkably, as shown in Figure 4A, no obvious electrochemical responses were observed for the other metal ions, compared with that obtained for Cu²⁺.

![Figure 3](image-url) **Figure 3.** (A) DPASV responses for (a) bare GC, (b) GC/APTMS, (c) GC/APTMS/CD, and (d) GC/APTMS/CD/TPEA electrodes in 10 mM PBS (pH 7.4) containing 5 μM Cu²⁺. (B) DPASV responses of the GC/APTMS/CD/TPEA electrode in aCSF (pH 7.4) containing Cu²⁺ (from a to m) 1, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, and 60 μM. Inset: Linear plot of peak current versus Cu²⁺ concentration. DPASV responses were recorded using a potential step of 5 mV, pulse width of 25 ms, pulse period of 100 ms, and pulse amplitude of 50 mV.

![Figure 4](image-url) **Figure 4.** (A, C, E) Selectivity and (B, D, F) competition experiments at the GC/APTMS/CD/TPEA electrode toward different physiological interferences. (A) Selectivity of metal ions against Cu²⁺. Ten μM for 1 pure Cu²⁺, 2 Fe²⁺, 3 Zn²⁺, 4 Ni²⁺, 5 Pb²⁺, 6 Cd²⁺, 7 Mn²⁺, 8 Co²⁺, 9 Fe³⁺ and 1 mM for 10 Ca²⁺, 11 Na⁺, and 12 Mg²⁺. (B) Competition experiment with the subsequent addition of 10 μM Cu²⁺ to the solutions containing other metal ions. (C) Selectivity of amino acids against Cu²⁺. Ten μM for 1 pure Cu²⁺, 2 Phenylalanine, 3 Methionine, 4 Glycine, 5 Glutamic acid, 6 Cystine, 7 Arginine, 8 Lysine Hydrochloride, 9 Leucine, 10 Serine, 11 Threonine, 12 Valine, 13 Isoleucine, and 14 Histidine. (D) Competition experiment with the subsequent addition of 10 μM Cu²⁺ to the solution containing amino acids. (E) Selectivity of biological species against Cu²⁺. Ten μM for 1 pure Cu²⁺, 2 UA, 3 AA, 4 DA, 1 mM for 5 Glucose, and 6 Lactate, 0.5 μM for 7 H₂O₂, 0.2 mM for 8 Oxygen. (F) Competition experiment with the subsequent addition of 10 μM Cu²⁺ to the solution containing biological species.
Furthermore, these potential metal ion interferences showed negligible effects on the electrochemical signal for Cu$^{2+}$ sensing (Figure 4B). The high selectivity of the present biosensor for Cu$^{2+}$ should be ascribed to the specific and stable affinity of TPEA toward Cu$^{2+}$.

Taking into account that amino acids in the biological system are capable of interacting with a lot of metal cations, several typical amino acids were also examined as potential interferences both in the selectivity and competition tests. As shown in Figure 4C, no electrochemical responses in the potential range of -0.3 to 0.2 V vs Ag/AgCl were observed at the GC/APTMS/CD/TPEA electrode in aCSF solution after the addition of the same concentration of amino acids as that of Cu$^{2+}$. On the other hand, the effect of all these amino acids on the electrochemical response to Cu$^{2+}$ was also investigated in detail. As shown in Figure 4D, negligible changes were observed when all the above compounds were added into the solution in the presence of Cu$^{2+}$. Moreover, the electroactive species which may endogenously exist in the rat brain, such as uric acid (UA), ascorbic acid (AA), dopamine (DA), glucose, lactate, H$_2$O$_2$, and O$_2$ were also examined. No electrochemical responses were observed at the GC/APTMS/CD/TPEA electrode in the tested potential range (Figure 4E). Meanwhile, the coexisting species did not cause interferences in determination of Cu$^{2+}$ either (Figure 4F). In order to imitate an environment like the rat brain, some other competition tests were also investigated. Negligible changes were observed when all the metal ions or amine acids or electroactive species were added into the solution in the presence of Cu$^{2+}$ (Figure S8, Supporting Information). All these selective experiments results indicate that our proposed electrochemical sensor could meet the selective requirements for environmental and physiological application. All the results indicate that the present GC/APTMS/CD/TPEA electrode shows high selectivity for Cu$^{2+}$ biosensing against other metal ions, amino acids, and biological species.

In addition, stability and reproducibility are also very essential for further application in a complex biological system. For the measuring stability test, the present GC/APTMS/CD/TPEA electrode was evaluated by performing 300 successive DPASV measurements in 10 mM PBS buffer (pH 7.4) containing 10 $\mu$M of Cu$^{2+}$. The relative standard deviation (RSD) of the peak currents obtained at the GC/APTMS/CD/TPEA electrode was 4.82%. On the other hand, for the storing stability test, the peak current showed no significant change after having been stored in darkness for one month at room temperature. These results indicated that the modified electrode was very stable and could be used for the continuous measurements.

To check the reproducibility, ten different GC/APTMS/CD/TPEA electrodes were prepared and used for the voltammetric measurement in the 10 mM PBS buffer (pH 7.4) containing 10 $\mu$M of Cu$^{2+}$. The relative standard deviation (RSD) of peak currents obtained at ten electrodes was calculated to be 5.07%, showing the high reproducibility of the present GC/APTMS/CD/TPEA electrode.

**Determination of Cerebral Cu$^{2+}$ in Rat Brain Microdialysates.** As demonstrated above, the developed electrochemical method for Cu$^{2+}$ detection with high selectivity and sensitivity substantially provided a direct platform for assaying Cu$^{2+}$ in the rat brain microdialysates, combined with in vivo microdialysis. As shown in Figure 5, after the addition of normal brain microdialysates (10 $\mu$L), the clear peak current was obtained at $-50$ mV vs Ag/AgCl, although no response was observed in pure aCSF. These results suggest the presence of Cu$^{2+}$ in the brain microdialysates. Furthermore, the peak current obtained at the GC/APTMS/CD/TPEA electrode remarkably increased with the addition of the brain dialysates followed by global cerebral ischemia, indicating the significantly increased concentration of Cu$^{2+}$. According to the calibration curve (inset in Figure 3B), the basal level of Cu$^{2+}$ in the brain dialysates was estimated to be 1.67 ± 0.56 $\mu$M, which was similar to the literature value reported by Ma and co-workers,$^{23a}$ but higher than other reports, possibly due to the different animal species used and different brain areas taken as samples.$^{23b−d}$ In addition, the concentration of Cu$^{2+}$ increased to 9.68 ± 0.53 $\mu$M after global cerebral ischemia. This is the first time that concentration of Cu$^{2+}$ in rat brain microdialysates after cerebral ischemia is reported.

The detected results of Cu$^{2+}$ in rat brain microdialysates by the developed method were compared with those obtained by using the traditional method ICP-AES. As summarized in Table 1, the concentrations of Cu$^{2+}$ determined by the present method were in good agreement with those obtained by the ICP-AES method. This comparison suggests that the developed electrochemical method based on the combination of designed molecule AE-TPEA and C-Dots has established a facile and direct approach for determining the concentrations of Cu$^{2+}$ and other metal ions in rat brain as well as investigating the role Cu$^{2+}$ and other metal ions play in brain chemistry.

**CONCLUSIONS** In summary, we have first designed and modified an electrochemical substrate with the combination of AE-TPEA and C-Dots. Based on the specific molecular recognition of TPEA toward Cu$^{2+}$, together with the amplified property of C-Dots, we have developed a direct, selective, and sensitive strategy for the electrochemical determination of Cu$^{2+}$. The developed method with theoretical simplicity, and less instrumental demands, has been successfully applied for the reliable detection of Cu$^{2+}$ in the rat brain. The simplicity in operation and instrumentation of this method, together with the characteristics of electrochemistry such as easy to real time and in vivo detection, should make it find further applications in biochemical investigations. This investigation has provided a methodology to design electrochemical detection of metal ions with high specificity and sensitivity as well as has opened a simple and reliable approach for monitoring cerebral species in the brain.
Table 1. Detected Results of Cu²⁺ in the Rat Brain Microdialysates by the Present Method and the ICP-AES Method

<table>
<thead>
<tr>
<th>Cu²⁺ (μM)</th>
<th>samples from normal rat brain</th>
<th>mean ± SD</th>
<th>samples from rat brain followed by ischemia</th>
<th>mean ± SD</th>
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<td>the present method</td>
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<td>1</td>
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