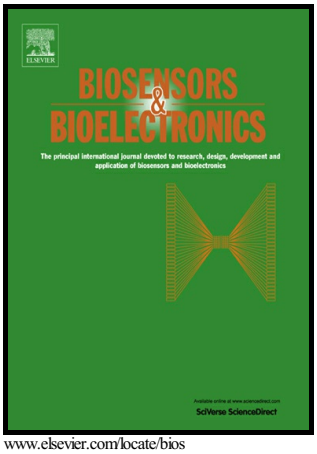

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Functionalized poly (ionic liquid) as the support to construct a ratiometric electrochemical biosensor for the selective determination of copper ions in AD rats

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Abstract

An efficient ratiometric electrochemical biosensor for Cu^{2+} determination was constructed using dual hydroxyl-functionalized poly (ionic liquid) (DHF-PIL) as the catalyst support. The DHF-PIL exhibited typical macroporous structure, which provided a high surface area of 39.31 m^2/g for the sufficient loading of biomolecules. The specific recognition of Cu^{2+} was accomplished by employing neurokinin B (NKB) for the first time, which could bind to Cu^{2+} to form a $[Cu^{II}(NKB)_2]$ complex with high specificity. Meanwhile, a common redox mediator, 2, 2'-Azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS) was modified into DHF-PIL by electrostatic interactions to act as an inner reference molecule, which provided a built-in correction for environmental effects and improving the detection accuracy. With this strategy, the developed electrochemical biosensor was capable of determining Cu^{2+} with a linear range between 0.9 and 36.1 μM and low detection limit (LOD) and quantificaiton limit (LOQ) of 0.24 and 0.6 μM, respectively. The sensor also displayed a satisfactory selectivity against a series of interferences in the brain, including metal ions, amino acids and other endogenous compounds. Accordingly, the present biosensor was successfully applied to evaluate Cu^{2+} levels in normal and AD rats.

Keywords: Functionalized PILs; Neurokinin B; Cu^{2+}; Electrochemistry; AD

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1. Introduction

It has been widely believed that copper ions (Cu^{2+}) play crucial roles in living systems as cofactors of numerous metalloenzymes that generate cellular energy, reduce molecular oxygen, and induce signal transduction (Bull et al., 1993; Gaggelli et al., 2006; Kang et al., 2014). However, if unregulated, the disturbance of the cellular Cu^{2+} homeostasis may lead to cell death and carious neurodegenerative diseases, including Alzheimer's disease (AD), Wilson's disease and Parkinson's disease (Taki et al., 2010; Valentine and Hart, 2003). The concentration of Cu^{2+} is found to be more than doubled in the cerebral amyloid deposits of AD brains compared with the neuropil of normal age-matched brains (Lovell et al., 1998; Smith et al., 1997), which might be responsible for inducing and enhancing the formation of the α-helix conformation of the alanine-based peptides by complexation with them (Zou and Sugimoto, 2000a, 2000b). It is, therefore, of considerable significance to establish highly sensitive and selective approaches for Cu^{2+} determination.

Up to now, a variety of strategies have been put forwarded for this purpose, including atomic absorption spectrometry (AAS) (Lin and Huang, 2001), inductively coupled plasma mass spectroscopy (ICP-MS) (Becker et al., 2007) and atomic emission spectrometry (ICP-AES) (Liu et al., 2005), fluorescence (Liu et al., 2016; Qing et al., 2015; Qu et al., 2012; Su et al., 2013; Vedamalai et al., 2014; Wang et al., 2010; Xiong et al., 2016; Zhu et al., 2012), immunoassay (Liu et al., 2013), colorimetric (Ding et al., 2016) and electrochemical approaches (Chai et al., 2013; Kimmel et al., 2012). However, those methods are usually complicated, time-consuming and costly. Fluorescence, although high sensitivity and specificity can be obtained with a number of well-designed fluorescent probes, including small organic molecules (Xiong et al., 2016; Zhu et al., 2012), metal nanoparticles and nanoclusters (Qing et al., 2015; Su et al., 2013; Vedamalai et al., 2014) and quantum dots (Liu et al., 2016; Qu et al., 2012), the susceptibility to the detection conditions often influences its fluorescence intensity and thus limits its wide application. Alternatively, electrochemical biosensors have been considered a promising technique because of their, simplicity, rapid response, and potential ability for real-time and on-site analysis (Yu et al., 2015), which are typically labeld with an electroactive species to generate corresponding electrochemical signals. For this application, specific recognition elements for Cu^{2+} should be designed for selectively capturing Cu^{2+} in the complicated in vivo environment. For instance, Tian's group has prepared a series of organic molecules, such as N-(2-aminoethyl)-N,N,N'tris(pyridin-2-ylmethyl)ethane-1,2-diamine and 2,2',2''-(2,2',2''-nitrilotris(ethane-2,1-diyl)-tris((pyridin-2-ylmethyl)azanediyl)triethanethiol, as well as the native Cu-free derivative of bovine erythrocyte copper-zinc superoxide dismutase (SOD-E_2Zn_2SOD) for specific recognitions of Cu^{2+} (Chai et al., 2013; Zhang et al., 2015; Zhu et al., 2012). However, the complicated synthesis procedure limits their further practical applications and in this regard, it is still of great necessity to look for a new and natural recognition biomolecule to construct an electrochemical biosensor, which is not only able to recognize Cu^{2+} with high selectivity, but also can fulfill the requirements of other analytical performances, particularly sensitivity and accuracy.

Porous polymers have received expanding interest, due to their low bulk density, extraordinary mechanical properties, variability of their monomer building blocks and the intrinsically charged characters (Li et al., 2010; Yang and Luo, 2014; Zhao et al., 2012). In particular, these polymers can serve as an immobilizing matrix for biomolecules in the biosensor system, which not only provide a suitable environment for firmly immobilizing biomolecules onto sensors through covalent binding at the functional sites, but also improve the loading capacity due to the existence of multiple pores (Rahman et al., 2005), enabling the realization of the signal amplification effect. Polymeric/polymerized ionic liquids (PILs) refer to a special type of polymers synthesized via polymerization of their ionic liquid monomers (IL) and differ

from conventional polyelectrolytes in that the cations and anions are usually organic and weakly coordinated (Zhang et al., 2011). In comparison with common polyelectrolytes, the physical properties of these PILs (e.g., solubility, polarity, etc.) can be altered more broadly and flexibly by counterion exchange (Zhao et al., 2012). The potential utility of PILs lies in the established tunability of the molecular structure of IL, leading to exquisite control over their physicochemical properties, in combination with the advantages of polymers: stability, processability and durability (Lee et al., 2012; Yuan and Antonietti, 2011). These characters make PILs versatile polymers for a multitude applications, including catalytic membranes, ionic conductive materials, carbon dioxide absorbents, microwave absorbing materials, stabilizing agent, and carbon precursors (Mao et al., 2015; Marcilla et al., 2006; Yuan and Antonietti, 2011; Yuan et al., 2010). It is worth mentioning that nanostructured PILs have received special interests due to their wide and potential range of applications, e.g., nanoparticles, nanosphere, nanogels, etc. For instance, Yuan's group has reported a highly ordered and tunable inner structure of PIL nanoparticles by precipitation polymerization from water with a much smaller diameter (20-40 nm) in the absence of external stabilizers (Yuan et al., 2011). Vancso et al. synthesized a new class of cross-linkable redox-responsive PILs with nanogels or macroscopic hydrogel networks, which was proved to be efficient dispersants in the microemulsion polymerization of methyl methacrylate (Sun et al., 2012).

In the present contribution, we reported on a facile method for constructing a novel macroporous dual hydroxyl-functionalized PIL (DHF-PIL)-based Cu^{2+} ratiometric electrochemical biosensor with high sensitivity and selectivity (Scheme. 1). The developed strategy possessed several features that made it particularly attractive for the detection of Cu^{2+}: (1) a macroporous DHF-PIL was synthesized with two hydroxyl functional groups at the alkyl side chains. The porous structure brought more binding sites to immobilize NKB, which was favorable for the permeation of biomolecules and thus facilitating the catalytic reaction; (2) for the first time, neurokinin B (NKB) was adopted for the specific biomolecular recognition of Cu^{2+} to form a $[Cu^{II}(NKB)_2]$ complex, which assured the selectivity of Cu^{2+} determination against a series of potential interferences in vivo; (3) an electroactive substance, 2, 2'-Azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS) was modified with DHF-PIL to serve as an inner reference moiety, which provided a reliable reference signal for avoiding environmental effects, thus improving the accuracy of the biosensor for the detection of Cu^{2+} in rat brain. Meanwhile, compared with the common ratiometric strategy that utilized two independent electrodes to construct a two-channel detection system, this method only involved one electrode, which was simpler to operate and more precise in keeping the electrode modification conditions exactly the same. Finally, the developed ratiometric electrochemical biosensor was successfully applied to determine Cu^{2+} in both normal and AD rat brains.

2. Materials and methods

2.1. Chemicals and Materials

1-vinylimidazole, 1-chloro-2,3-propanediol, 2,2'-azobis(2-methylpropionitrile) (AIBN), tetraethyl orthosilicate (TEOS), methyl methacrylate (MMA), trihydroxymethyl propane triacrylate (TMPTA) and all the tested metal ions were bought from Aladdin-Reagent Company (Shanghai, China). Neurokinin B (NKB), 2,2'-Azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS), glutaraldehyde (25%, Glu), glucose (Glu), lactate (Lac), uric acid (UA), ascorbic acid (AA), dopamine (DA), all the amino acids and copper sulfate ($CuSO_4$) were all purchased from Sigma-Aldrich (USA) and used without further purification. Phosphate buffer saline (PBS, 0.1 M, pH 7.4) containing 8.6 mM $Na_2HPO_4 \cdot 12H_2O$, 1.88 mM NaH_2PO_4, 50.9 mM NaCl and 2.94 mM KCl was employed as the detection electrolyte. Water (≥ 18 M) used throughout the whole experiment was purified with Millipore system. All other reagents were of at least

analytical grade and commercially available. The cerebrospinal fluid (CSF) and hippocampus homogenate samples from normal and AD rats were kindly provided by L. Zhang in other group.

2.2. Instruments

All the electrochemical experiments were carried out on CHI 832D electrochemical workstation (Shanghai Chenhua Instrument, CHI, China). A conventional three-electrode system composed of a platinum wire as counter electrode, a saturated calomel electrode (SCE) as reference electrode, and a bare or a modified glassy carbon electrode (GCE) as working electrode. Unless otherwise indicated, all electrochemical measurements were performed in the presence of oxygen in a reaction mixture.

^{1}H NMR and mass spectrum (MS) were taken for the structure confirmation of the IL monomer. ^{1}H NMR spectra were measured on a Bruker DMX 400 spectrometer using $SiMe_4$ as an external standard at 25 °C. Scanning electron microscope images (SEM, Hitachi Co. Ltd., Tokyo, Japan) were taken using a field emission gun Hitachi S-4800 scanning electron microscope (operating at 1 kV) for morphological analysis of DHF-PIL and electrodes. X-ray diffraction (XRD) was measured by a Rigaku XRD-Ultima IV (Japan) using Cu Kα radiation (λ = 0.154 nm). N_2 sorption experiments were performed with a Quantachrome Autosorb 3B. The specific surface area and pore volume were calculated using Brunaer–Emmett-Teller (BET) equation and the Barrett-Joyner-Halenda (BJH) method, respectively. Pore size distribution was calculated by density functional theory (DFT) method. X-ray photoelectron spectroscopy (XPS) investigation was carried out on an ESCALab MKII X-ray photoelectron spectrometer using Mg Kα radiation. Inductively coupled plasma mass spectroscopy (ICP-MS) was conducted by a Hitachi P-4010 system.

2.3 Preparation of the macroporous poly (ionic liquid), DHF-PIL and DHF-PIL-ABTS

Synthesis of the silica colloidal crystal templates, ionic liquid monomer (IL-Cl) and poly (ionic liquid) were available in Supporting Information.

For the preparation of the DHF-PIL-ABTS composite, 500 μL DHF-PIL suspension (1 mg/mL) was incubated with 500 μL ABTS (1 mg/mL) for 3 h under 25 °C and 250 rmp. After centrifugation under 1200 rmp for 10 min, the upper supernatant was discarded and the remaining was re-dissolved with deionized water and centrifuged for three times. When in use, the obtained DHF-PIL-ABTS composite was dispersed in ethanol with the final concentration of 1 mg/mL.

2.4 Fabrication of the ratiometric DHF-PIL-ABTS/NKB/Glu biosensor

Before modification, the bare glassy carbon electrode (GC) was pre-cleaned using acetone, ethanol and distilled water with ultrasonication. To prepare the bioanalytical system, firstly, 10 μL of the as-prepared DHF-PIL or DHF-PIL-ABTS suspension (1 mg/mL) was drop-casted onto the cleaned bare electrode surface with a micropipet, which was then allowed to dry in ambient temperature for 1 h. This electrode was denoted as DHF-PIL or DHF-PIL-ABTS electrode. After washed carefully with distilled water, 5 μL of NKB solution in PBS (0.1M, pH 7.4) (0.002 mg/mL) with negative charge was introduced onto the DHF-PIL or DHF-PIL-ABTS layer on the basis of the static interaction with DHF-PIL or DHF-PIL-ABTS to specifically recognize Cu^{2+}, which was then denoted as DHF-PIL/NKB or DHF-PIL-ABTS/NKB electrode. In the last step, the electrode was further casted with 2 μL of 0.1% glutaraldehyde (Glu) to stabilize the formed layers and enlarge the connecting areas between DHF-PIL-ABTS and NKB. Following preparations, the modified sensor was rinsed with distilled water prior to determination. The finished DHF-PIL /NKB/Glu or DHF-PIL-ABTS/NKB/Glu electrode should be stored in 4°C when not in

use. In the same way, DHF-PIL electrode, DHF-PIL-ABTS electrode and NKB electrode were prepared for comparisons.

2.5 Electrochemical sensing procedure

The electrochemical sensing of Cu^{2+} was performed at room temperature in a PBS (0.1M, pH 7.4) buffer solution. In a typical run, different concentrations of Cu^{2+} standard solution or real samples from rats containing Cu^{2+} were added to 5 mL PBS buffer, followed by gentle stirring for a certain time. The differential pulse voltammetry (DPV) signals were recorded by the potential scan in the range of -0.3 to 0.7 V vs SCE at 100 mV s^{-1}. The quantification of Cu^{2+} was evaluated by calculating the ratios of current intensity between reduction peaks at -0.05 V for Cu^{2+} and 0.5 V for the reference substance ABTS.

3. Results and discussion

3.1 Characterizations of the macroporous DHF-PIL nanocomposite

In this work, after completing the polymerization, the obtained DHF-PIL suspension was modified with the negative charged ABTS by their electrostatic interactions to form a DHF-PIL-ABTS composite. On one hand, the presence of the conductive ABTS facilitated the electron transfer on the electrode, which was beneficial for enhancing the sensitivity. On the other hand, ABTS was redox-active and its characteristic reduction peak in the voltammetry curve could provide a reliable reference signal for avoiding environmental effects, thus improving the accuracy of the determination. We measured the zeta-potentials of DHF-PIL and DHF-PIL-ABTS, which were + 42.3 mV and +21.2 mV, respectively, indicative of the successful conjugation of ABTS with DHF-PIL.

XRD result from Figure S2 shows that the DHF-PIL is non-crystalline. Figure 1 displays the morphology of DHF-PIL by SEM and N_2 sorption techniques. The porosity of DHF-PIL can be reflected from the N_2 sorption measurements in Figure 1a and b, which indicates the existence of macropores in DHF-PIL with the average pore diameter 62 nm. The specific Brunauer−Emmett−Teller (BET) surface area and pore volume were calculated to be 39.31 m^2/g and 0.5 cm^3/g, respectively. The high surface area would be indispensable and advantageous for improving the sensitivity in later research. Consistent with the N_2 sorption results, the inserted SEM image in Figure 1a also reveals the distinctive morphology of macroporous DHF-PIL.

3.2 Cu^{2+} determination by the fabricated DHF-PIL-ABTS/NKB/Glu biosensor

Differential pulse voltammetry (DPV) was employed for Cu^{2+} determination. As presented in Figure 2a, only one reduction peak located at 0.5 V *vs* SCE is appeared at DHF-PIL-ABTS electrode when 9 μM Cu^{2+} is contained in the PBS buffer, while no obvious peaks are observed at other electrodes, e.g., bare GC and DHF-PIL electrode in the presence of Cu^{2+} with the same concentration. This cathodic peak can be ascribed to the electrochemical reduction of ABTS motif on DHF-PIL. After a certain amount of NKB solution was casted onto the DHF-PIL-ABTS film, besides the ABTS reduction peak at 0.5 V, a new reduction peak at around -0.05 V is observed in the voltammogram, which corresponds to the reduction reaction of Cu^{2+} recognized by NKB at the DHF-PIL-ABTS/NKB electrode surface. Consequently, the quantification of Cu^{2+} in this ratiometric assay can be realized on the basis of the current ratios (I_P / $I_{P(R)}$) between the two peaks at -0.05 and 0.5 V, in which the current at -0.05 V for Cu^{2+} was denoted as I_P, and $I_{P(R)}$ represented the currents of ABTS at 0.5 V. Meanwhile, in this work, DHF-PIL and ABTS were employed as the substrate, which was designed for massive loading of NKB to specifically recognize Cu^{2+} and

electron-transfer acceleration to achieve a high sensitivity. As a control, we compared the electrochemical responses toward Cu^{2+} among three electrodes, e.g., NKB, DHF-PIL/NKB and DHF-PIL-ABTS/NKB/Glu modified electrodes. Figure 2b displays that without DHF-PIL or DHF-PIL-ABTS modifications, a single NKB layer on the surface of bare electrode leads to an obvious decline in the reduction current of Cu^{2+}, accompanied with the negative-shifted and widened irregular reduction peak. By contrast, when DHF-PIL or DHF-PIL-ABTS layers were dropped onto electrodes, the reduction currents toward Cu^{2+} increase significantly compared with single NKB modification, along with a more well-defined reduction peak at -0.05 V. The specific electroactive surface area for the above-mentioned three electrodes can be calculated according to the Randles-Sevcik equation (Sun et al., 2012):

$$I_p=2.69\times10^5\ AD^{1/2}\ n^{3/2}\ v^{1/2}C$$

which is 0.35, 0.27 and 0.73 (10^{-6} cm^2), respectively. As expected, the sensitivity for Cu^{2+} determination on DHF-PIL-ABTS/NKB/Glu electrode is appreciably improved by 2~3 folds compared with other two electrodes. Such a result was adequate enough to attest that the participation of ABTS with DHF-PIL was favourable for Cu^{2+} determination due to the amplified property in itself and the strategy of using NKB as the specific recognition element for Cu^{2+} was also achievable.

3.3 Selectivity of the DHF-PIL-ABTS/NKB/Glu electrode for Cu^{2+} determination

The data presented above demonstrated that our method is a simple and efficient way to monitor Cu^{2+}. The complexity of the brain environment puts forward a great challenge for *in vivo* detection of Cu^{2+}. Therefore, the selectivity of the DHF-PIL-ABTS/NKB/Glu electrode for Cu^{2+} was evaluated by monitoring the $I_P/I_{p(R)}$ values in PBS solution containing other metal ions (Ca^{2+}, Fe^{3+}, Mg^{2+}, Na^{+}, Zn^{2+}, Fe^{2+}, Mn^{2+}, Cu^{+}, Cd^{2+}, Pb^{2+}, Ni^{2+} and Co^{2+}), amino acids (Cys, Phe, Met, Gly, Glu, Arg, Lys, Leu, Ser, Thr, Val, His) and several representative co-existing species (AA, DA, UA, Glu, Lac). As shown in Figure S7, remarkably, no obvious currents were observed at the reduction peak of -0.05 V upon additions of these mentioned interferences (a, c, e), which can be reflected from the dramatic decrease in the $I_P/I_{p(R)}$ ratios compared with that of Cu^{2+}. On the other hand, for the competition tests, as revealed in Figure S7b, d and f, the $I_P/I_{p(R)}$ values are almost unperturbed in the simultaneous presence of these species and Cu^{2+} relative to that of Cu^{2+}. Although Cys has been reported to be able to combine with Cu^{2+}, it is possible that after reaction with NKB, Cu^{2+} could not be released from the $[Cu^{II}(NKB)_2]$ complex, and in this case, Cys would not be accessible to combine with Cu^{2+}. All these evidences indicate the excellent selectivity of the present method for Cu^{2+} against metal ions, amino acids and other endogenous compounds in the cerebral system, which is own to the specific interaction of NKB with Cu^{2+}.

3.4 Sensitivity, stability and reproducibility of the DHF-PIL-ABTS/NKB/Glu electrode for Cu^{2+} determination

To assess the sensitivity of the proposed electrochemical assay, routine samples of Cu^{2+} with different concentrations using the developed DHF-PIL-ABTS/NKB/Glu electrode were measured. As demonstrated in Figure 3, the $I_P/I_{p(R)}$ ratios increase with the increment of Cu^{2+} concentrations in the standard solution. Under the optimized conditions, the linear range of Cu^{2+} at the DHF-PIL-ABTS/NKB/Glu electrode is between 0.9 and 36.1 μM. In consideration of the wide

span of Cu^{2+} levels between normal and AD rats, it would be more ideal for practical applications to have a broader range of dynamic responses. The linear dependencies of Cu^{2+} concentrations yield the regression equation of $I_P/I_{P(R)}$ = (0.40 ± 0.01) $C_{(\mu M)}$ + (0.76 ± 0.13) with a correlation coefficient *R* of 0.9988. According to IUPAC recommendations (IUPAC, 1976), the detection limit (LOD) and quantification limit (LOQ) of this approach were calculated to be 0.24 and 0.6 μM, estimated from 3σ and 10σ of the baseline signals, respectively. This LOD value is equal to or better than that of other reported Cu^{2+} detection assays (Su et al., 2013;Kang et al., 2014; Koneswaran and Narayanaswamy, 2009; Lan et al., 2010; Liu et al., 2016; Zheng et al., 2010; Zhu et al., 2012), which indicates that the established electrochemical strategy holds great potential for Cu^{2+} determination in a biological matrix, benefited from the porous DHF-PIL nanomaterial and the highly specific recognition ability of NKB to Cu^{2+}. A table has been added to more clearly compare our work with other published methods (Table S1).

The calculated precision of the methodology with relative standard deviation of 6.2% for consecutive measurements of 10 μM Cu^{2+} in one day (n=6) indicates an acceptable reproducibility. After the DHF-PIL-ABTS/NKB/Glu electrode was determined in PBS for four days and 3~4 h every day, 91.1% of its initial current response was retained, which further demonstrates a reliable stability of the detection signal using the fabricated assay.

3.5 Quantitative estimation of Cu^{2+} levels in the cerebrospinal fluid and hippocampus of normal and AD rats

These initial results validate the efficiency of our electrochemical probe to determine Cu^{2+} in real biological samples. Figure 4 displays the DPV responses of Cu^{2+} obtained at DHF-PIL-ABTS/NKB/Glu electrode in the cerebrospinal fluid (CSF) of normal (solid line) and AD (dashed line) rat brain using standard addition method. As is seen, the electrochemical signals from CSF differ greatly between normal and AD rats, indicating the variations in the levels of Cu^{2+} associated with AD. Based on our postcalibration, the basal Cu^{2+} concentration in rat CSF was estimated to be 1.21 ± 0.25 μM (n = 3). A clear rise of this value up to 8.15 ± 1.72 μM (n = 3) is witnessed in the CSF of AD rats ($P < 0.01$). In the same way, we inspected the variations of Cu^{2+} concentration in the hippocampus of normal and AD rats and the results are summarized in Table 1, which shows a similar tendency to that in CSF ($P < 0.01$). These results are in good agreement with previously reported results (Ma et al., 1999; Tian et al., 2002).

In the end, we also compared the data by the present electrochemical method with those using the standard ICP-MS technique to evaluate the accuracy the ratiometric DHF-PIL-ABTS/NKB/Glu electrode for Cu^{2+} determination. It is clear to observe from Table 1 that both of the absolute values and the variations of Cu^{2+} concentration in normal and AD rats using the two assays are rather similar. This comparison signifies that the developed electrochemical assay based on the specific binding between NKB and Cu^{2+} opens up a reliable *in vivo* approach for determining the concentrations of metal ions in rat brain.

4. Conclusions

It was the first time for us to initially develop a direct and simple electrochemical method for the determination of Cu^{2+} in normal and AD rats based on the biomolecular recognition of NKB immobilized onto the porous and functionalized polymerized ionic liquid. The porosity of the polymer brought more binding sites to immobilize biomolecules onto sensors and favorable for the permeation of biomolecules,

hence facilitating the catalytic reaction and endowing the method with acceptable sensitivity. Physiological levels of interferences didn't present obvious signals at the fabricated biosensor. The accuracy of this method was guaranteed by incorporating ABTS into polymer to construct a ratiometric electrode. Compared with other established approaches for Cu^{2+} determination, including the standard ICP-MS method, our strategy retained the powerful features of ICP-MS (e.g., sensitive, selective and accurate), and also offered other additional advantages, such as faster in analysis, simpler in operation and lower demands in instrument. The proof-of-concept experiment demonstrated that electrochemical technique can potentially serve as a viable alternative for facile and sensitive clinical analysis of important biomarkers related to neurodegenerative diseases such as AD, Huntington's disease and Parkinson's disease.

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Figure captions

Scheme 1. Electrochemical sensing for Cu^{2+} based on the ratiometric DHF-PIL-ABTS/NKB/Glu biosensor.

Figure 1. (a) N_2 sorption of isothermals of DHF-PIL; (b) Pore size distribution curve of DHF-PIL. Inset is the SEM image of the DHF-PIL with the scale bar of 500 nm.

Figure 2. (a) DPV curves obtained at bare GC, DHF-PIL, DHF-PIL-ABTS and DHF-PIL-ABTS/NKB/Glu modified electrodes in PBS buffer solution containing 9 μM Cu^{2+}; (b) DPV responses of NKB, DHF-PIL/NKB and DHF-PIL-ABTS/NKB/Glu modified electrodes in PBS buffer solution containing 9 μM Cu^{2+}.

Figure 3. DPV curves of the DHF-PIL-ABTS/NKB/Glu electrode toward successive additions of Cu^{2+} at the concentrations of 0.9、1.6、2.6、3.8、5.2、6.9、8.9、11.2、13.8、16.7、19.9、23.4、27.2、31.5 and 36.1 μM. Inset is the corresponding linear plot of the $I_P/I_{p(R)}$ ratios versus the absolute concentrations of Cu^{2+}.

Figure 4. DPV responses of Cu^{2+} in the CSF of normal (solid line) and AD rat brains (dashed line) on the DHF-PIL-ABTS/NKB/Glu electrode.

Table 1. Results of Cu^{2+} determination by the present electrochemical assay and ICP-MS method in the CSF and hippocampus of normal and AD rats (Mean ± SD, n=3).

Scheme 1

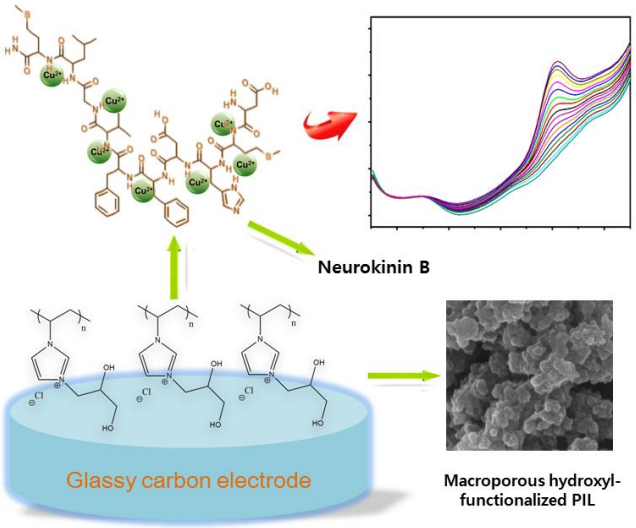

Figure 1

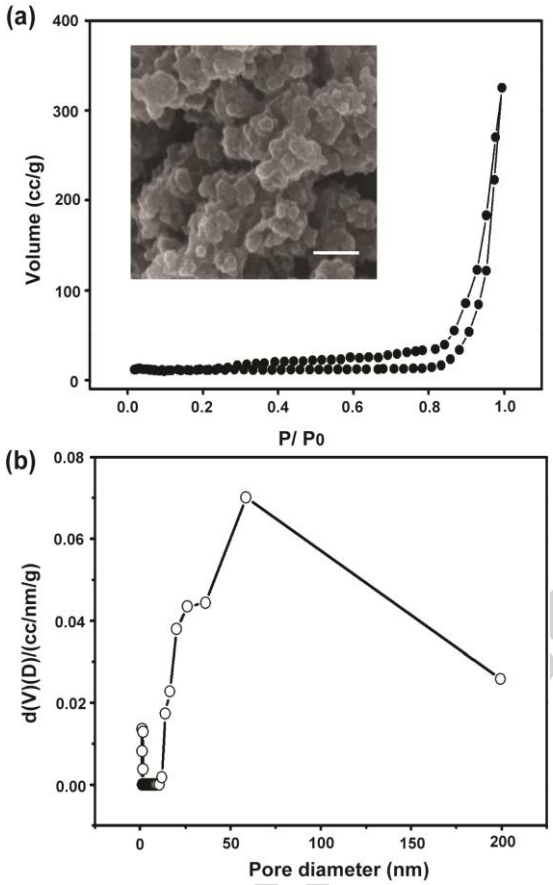

Figure 2

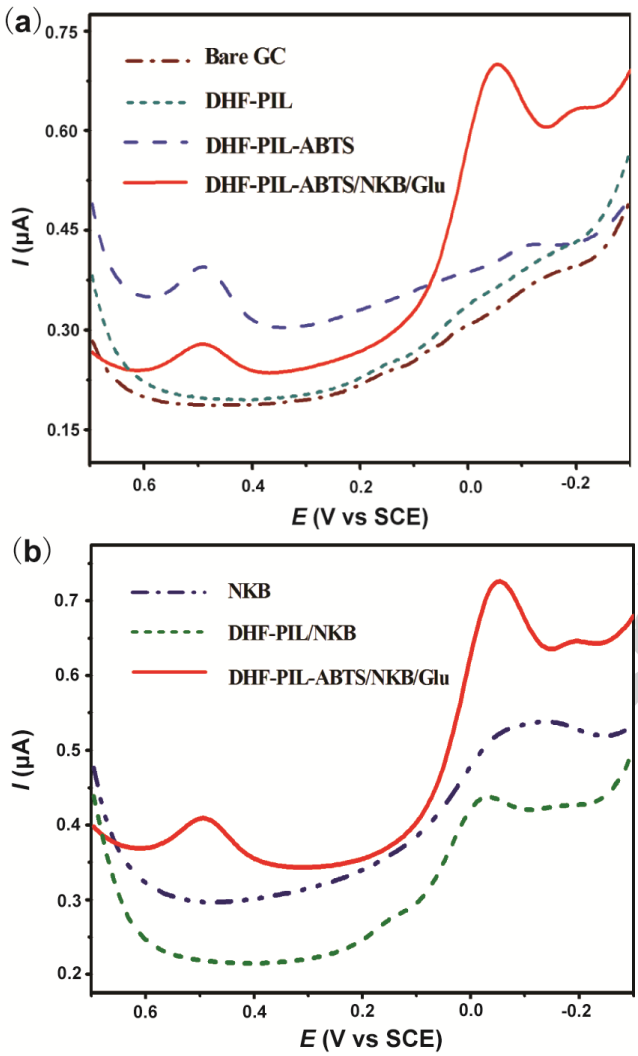

Figure 3

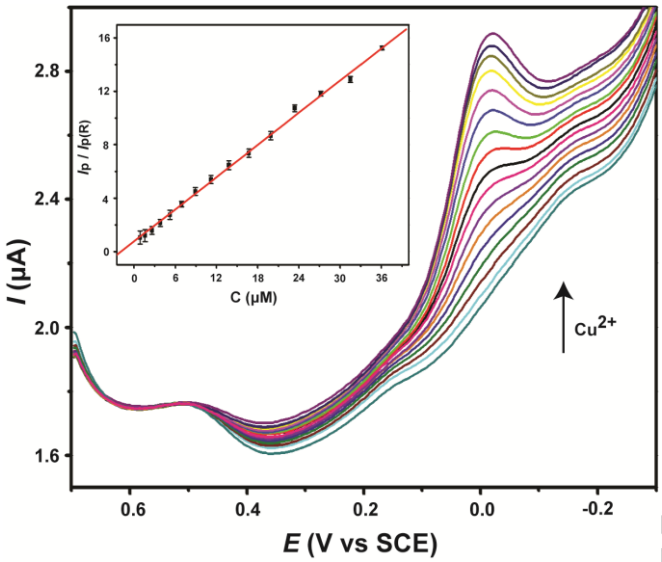

Figure 4

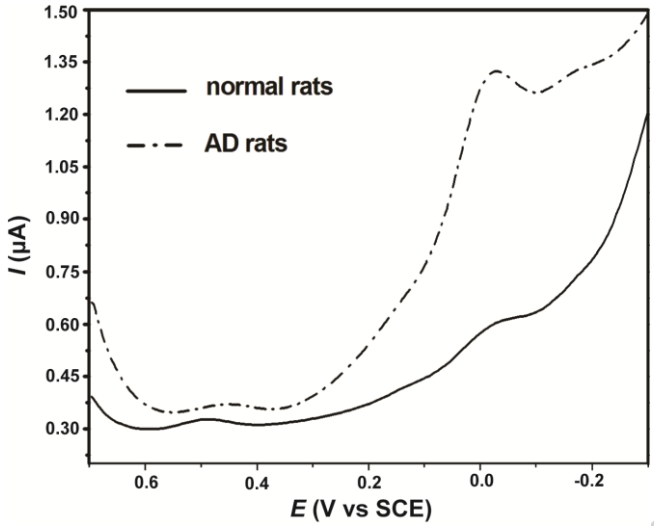

Table 1 Results of Cu^{2+} determination by the present electrochemical assay and ICP-MS method in the CSF and hippocampus of normal and AD rats (Mean ± SD, n=3)

	Cu^{2+} concentration (uM)	Normal group	AD group
Electrochemical method	CSF	1.21 ± 0.25	8.15 ± 1.72
	Hippocampus	1.27 ± 0.22	7.21 ± 0.77
ICP-MS method	CSF	1.32 ± 0.18	8.34 ± 0.85
	Hippocampus	1.34 ± 0.17	7.09 ± 0.79

highlights

- A sensitive electrochemical method for the detection of Cu^{2+} was presented.
- Neurokinin B was employed for the specific recognition of Cu^{2+}.
- Polymerized ionic liquid was adopted for neurokinin B loadings.
- This methodology realized the assessment of Cu^{2+} levels in AD rat brains.