Ferrum nano particles and multiwall carbon nano tubes based electrode as FIA detector for determination of amino acids in hypothalamus microdialysis fluids

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Abstract. An amperometric electrode based on multiwall carbon nanotubes (MWCNTs) and Fe nanoparticles (NPs) has been successfully fabricated. Combined with Flow Injection Analysis (FIA) and chromatography separation column, the electrode exhibits linear response in the concentration range of 0.1 -12 μM and the sensitivity of 30.0 nA μM⁻¹ for most of amino acids. The determination of 17 amino acids in the hypothalamus microdialysis fluids of guinea pigs, illustrates that the electrode is a powerful tool to investigate physiology and pathology mechanisms

1. Introduction
Amino acids (AAs) are essential building blocks of biological molecules and play key roles in many neurochemical response mechanisms such as memory, appetite control, and pain transmission. The accurate determination of amino acids can improve our understanding of its biological role and facilitate the treatment of diseases caused by its disruption. Because most of amino acids lack natural chromophore or fluorophore for direct photometric and fluorometric detection, derivatization procedures are necessary for the sensitive and selective determination. However, derivatization procedures generally suffer from the instability of the derivatization product and time-consuming. The presence of relatively low concentrations of amino acids in microdialysis samples requires a sensitive analytical method for their determination. Microdialysis is a powerful technique which permits determination of the extracellular concentration of amino acid neurotransmitters during various pharmacological or physiological manipulations. The amperometric electrode detector combined with FIA possesses many advantages such as high sensitivity, rapid response, and simplicity in operating procedure, and much attention has been paid to the development of electrochemical detection of amino acids. However, many amino acids are nonelectroactive at the conventional carbon electrodes. The noble metal electrodes exhibit a greater degree of activity toward amino acids oxidation, but they are subject to rapid surface fouling [1]. The alternative method is using non-noble transition metal, such as nickel and copper [2], chemically modified electrodes that can mediate fast electron transfer between amino acids and the substrate electrode and can reduce surface fouling. These modifiers include copper nanoparticles and zinc oxide nanorod array [3], copper–manganese alloy [4], Cu₂V₂O₇, nickel–copper alloy [5], nickel hydroxide nanoflakes [6], copper microparticle [7], Cu₂O [8], cobalt and cadmium doped nickel hydroxide [9], and nickel–curcumin complex [10]. Furthermore, carbon nanotubes (CNTs) are of good electrocatalytic properties as electrode materials. Iron is also a
transitional element, should possess similar properties with nickel and copper, but rarely reported. Fe nanoparticles (NPs) are complex centre with AAs like Cu NPs, while MWCNTs can disperse iron nanoparticles to avoid reunion and fleetly transfer electrons from AAs to the electrode. The bulk sensing layer is convenient for repeatable renew of electrode. Thus, Fe NPs and MWCNTs were employed to fabricate a novel AAs sensor. The electrochemical behaviour of Fe NPs/MWCNTs electrode and the oxidation of AAs’ complex with Fe NPs were investigated by electrochemistry methods. Moreover, the electrode combined with FIA method and separation technology was proposed for the determination of AAs with fast response, good stability, and sensitivity. The highlights of this work are that the electrode proposed used an electrolyte with near neutral pH value (8.0) and applied low work potential (0.6 V) as well as determined successfully 17 amino acids in the hypothalamus micro-dialysis fluid of guinea pigs.

2. Experimental

2.1. Reagents and animals
MWCNTs (GMWCNT2, 10–20 nm diameter, 10–20 µm length, purity > 99.9%) were purchased from Shanghai Jikart Chemical engineering Sci & Tech Co. Ltd. (Shanghai, China) and Nano-Fe (50 nm average diameter, purity 99.99%) was purchased from Beijing Deke Daojin Science & Technology Co. Ltd (Beijing, China). They were used without further purification. Adult guinea pigs weighing 400–500 g purchased from the Experimental Animal Centre of Medical School, Shanghai Jiao Tong University. All amino acids were obtained from Sigma Co. (USA). Other reagents were of at least analytical-reagent grade. All solutions were prepared using doubly distilled water. Standard stock solution of amino acids (20 mM) were prepared in a mixture of water–methanol (1:1) and stored at 4°C for 1 month. Working standard solutions were prepared daily by diluting the stock solutions with PBS buffer (pH 8.0).

2.2. Measurement
Electrochemical experiments were performed on a CHI 832A workstation (CH Instruments, Shanghai, China) at room temperature. A conventional three electrode system was employed, including a homemade Fe–MWCNTs electrode (3.0-mm-diameter) as a working electrode (WE), an Ag/AgCl reference electrode (RE), and a platinum wire counter electrode (CE). All of the potentials quoted in this work were referred to SCE. The FIA system was consisted of a Cole–Parmer microprocessor pump drive, a Rehodyne model 7125-sample injection valve (20 mL loop) with interconnecting Teflon tube and a Zensor SF-100 thin layer detecting electrochemical system (The dimension of the electrode was specifically designed for it). Chromatographic separations were performed using a silica based HPLC column (Prevail organic acid 5 μ, Alltech, 100×4.6 mm) with a mobile phase of 0.1 M, pH 8.0 PBS.

Acquity UPLC system (Waters Co.) with a Series 200 IC pump and a diode array detector 235C (Perkin Elmer, Norwalk, CT) was also applied to determine amino acids. The column used was Shim-pack C18, 5 μm, 150 mm×4.6 mm (Shimadzu, Japan). The micro-dialysis fluids were derived with 6-aminoquinolyl-N-hydroxysuccinimidylcarbamate (AQC) reagent and the derivatives were separated by a two gradient elution and detected by fluorescence detector. The wavelength used for the measurement of the derivatives was 286 nm. The HPLC flow rate was 1.5 ml/min. All the measurements were performed at room temperature. Comparison between results of two analysis methods was realized.

The experiment system was shown in Figure 1. It consists of three parts: A) microdialysis, B) AAs separation column, and C) Flow Injection Analysis (FIA) with Fe NPs/MWCNTs electrode as a detector. Fe nanoparticles and MWCNTs with weight ratios (W_{Fe}/W_{Fe+CNTs}) of 0.30, 0.25, 0.20, 0.15 and 0.10 were mixed and grind in a mortar to obtain a homogeneous mixture. The Fe-MWCNTs electrode was prepared by mixing the mixture and silicone oil in a ratio of 3:2 (w/w) in a mortar and grind until a uniform paste was obtained. A portion of the resulting paste was packed firmly into the
cavity of a polytetrafluoroethylene (PTFE) tube (2 mm depth, 3.0 and 1.0 mm diameter for electrochemical and application investigation, respectively). The electric contact was established via a copper wire with the aid of Wood’s alloy as shown in the inset of Figure 1C. The electrode surface was gently smoothed by rubbing on a piece of weighing paper just prior to using. This procedure was also used to regenerate the surface of the electrode proposed. SEM image of the surface of spherical Fe/MWNTs composite structure bulk was shown in Figure 2.

**Figure 1.** Schematic diagram of the electrochemical measurement system with Fe NPs/MWCNTs electrode for determining amino acids in the hypothalamus microdialysis fluids of guinea pigs. It included A) microdialysis, B) AAs separation column, and C) Flow Injection Analysis (FIA) with Fe NPs/MWCNTs electrode as a detector (a larger view is above).

**Figure 2.** SEM image of the surface of the electrode proposed. It is evident that the MWCNTs are very long and present as a highly entangled network structure and the images reveal that the Fe NPs were distributed randomly and uniformly on the surface of CNTs. The MWCNTs in the modified electrode play an important role to disperse Fe NPs and enhance conductivity of the electrode.

**Figure 3.** Schematic diagram of electrochemical reaction of the Fe NPs/MWCNTs electrode. The negative charged AAs- molecular (generator) were adsorbed on Fe NPs (acceptor) with positive charge due to electrostatic interaction. An electron can fleetly be transferred from AAs to the electrode and formed electrochemical current.
2.3. Sample treatment
Guinea pigs were narcotized by 3 g/dl pentobarbital of intraperitoneal injection and located on the brain locator. The dialysis micro-needle was imported into a predetermined depth of hypothalamus and fixed by denture acrylic. A Ringer’s perfusion fluid was perfused via the needle at 20 μl/min. Different period of dialysates were collected, respectively. All procedures of animal experiments complied with the regulations of the “protection of animals used for experimental and other scientific purposes” from the relevant directives of Good Laboratory Practices (GLP). The study protocols were approved by the Ethical Committee at our institutes.

2.4. Sensing mechanism of Fe-MWCNTs electrode
Isoelectric points of most AAs are smaller than 8, so they show negative charged in PBS of pH 8.0. Positive charged Fe NPs at the surface of the electrode interact with AAs through the electrostatic interaction. Thus Fe NPs at the surface of the electrode become the complex center. The MWCNTs in the modified electrode play an important role to disperse Fe NPs and enhance conductivity of the electrode. It can fleetly transfer electron from AAs to the electrode. Sensing mechanism of Fe-MWCNTs electrode was shown in Figure 3.

3. Results and Discussion
3.1. Characterization of Fe-MWCNTs electrode
The dependence of response current on the ratio of W_{Fe}/W_{Fe+CNT} was tested by using linear sweep voltammetry, as shown in Figure 4. The response current increased with increasing of Fe nanoparticles proportions until the ratio reached 25%, because of the increase of the complexation center. When the ratio was 30%, the response current became smaller (second-highest in Fig.4). Therefore, 25% of ratio was used in the following experiments.

**Figure 4.** Linear sweep voltammetry curves of 2 mM glycine at the electrode with different ration of W_{Fe}/W_{Fe+CNT} in pH 8.0 PBS. Scan rate: 100 mV/s. The response current increased with increasing of Fe nanoparticles proportions until the ratio reached 25%. When the ratio was 30%, the response current became smaller. Therefore, 25% of ratio was used in the following experiments.

**Figure 5.** The peak current I_p and peak formal potential E° of Fe-MWCNT electrode were depended on the pH value of the PBS. With the increase of pH value of the solution, both anodic peak current, I_p, and cathode peak current, I_p, were enhanced. The peak formal potential E° of the redox couple was negatively shifted. Scan rate: 100 mV/s.

3.2. Effect of pH on the Sensor Response
To investigate the influence of pH value on the voltammetric behaviour of glycine on Fe–MWCNTs electrode, the electrode was examined in a pH range from 7.0 to 10.5, and the results were shown in
Figure 5. As shown, with the increase of pH value of the solution, both anodic peak current $I_{pa}$ and cathode peak current $I_{pc}$ were enhanced and the formal potential $E^0$ of the redox couple negatively shifted, suggesting that OH participated in the redox process of the modified electrode.

3.3. Cyclic Voltamograms
The cyclic voltammograms of glycine on Fe-MWCNTs electrode was shown in Figure 6. The oxidation and reduction peaks of glycine appear at 0.6 and 0.08 V, respectively. The peak currents has a linear relationship with the concentration of glycine in a range from 0.1 to 20 mM.

3.4. Chronoamperogram and Calibration curve
Figure 7 represents the chronoamperogram of the electrode obtained in 0.1 M PBS solution (pH 8.0) at 0.6 V by successively adding aliquots of glycine. The sensor proposed displayed a fast amperometric response time of less than 2.5 s. The calibration curve is also shown as the inset (linear response range: 0.1 - 20 mM, sensitivity: 1.1 $\mu$A mM$^{-1}$, RSD: 3.5%).

3.5. Stability and Reproducibility
Table 1 shows that the response current a little increased when the electrode was renewed while a slightly decreased after storage for 3 months. The possible reason is Fe NPs on the surface of the electrode was partly oxidized, resulting decrease of the complexation center of AAs. Five Fe–MWCNTs electrodes were fabricated, and the RSD for the individual determination of 0.5 mM glycine was 3.8%. The long-term stability of the modified electrode was tested after being stored in dry conditions at room temperature for 60 days, and no significant change in current responses was observed. Thus, the Fe–MWCNTs electrode exhibited acceptable stability and reproducibility.
Table 1. Measurement precision for the sensor in the buffer solution (pH 8.0) including 0.5 mM of glycine \((n = 11)\). R.S.D.: relative standard deviation.

| Times | Response/\(\mu A\) After renew | Response/\(\mu A\) After storage for 3 months |
|-------|-------------------------------|-----------------------------------------|
| 1     | 2.31                          | 2.20                                    |
| 2     | 2.44                          | 2.10                                    |
| 3     | 2.23                          | 2.25                                    |
| 4     | 2.34                          | 2.27                                    |
| 5     | 2.38                          | 2.22                                    |
| 6     | 2.40                          | 2.15                                    |
| 7     | 2.24                          | 2.13                                    |
| 8     | 2.22                          | 2.09                                    |
| 9     | 2.41                          | 2.30                                    |
| 10    | 2.39                          | 2.15                                    |
| 11    | 2.25                          | 2.12                                    |
| Average/\(\mu A\) | 2.33                          | 2.18                                    |
| RSD\(^a\) | 3.5%                          | 3.3%                                    |

3.6. Optimal experiment conditions of FIA

Figure 8. When FIA system with the electrode proposed as a detector, responses of a series of glycine concentration (0.1-12 \(\mu M\)) with 250 \(\mu l/min\) of flow rates in pH 8.0 of PBS carrier solution. A set of 11 replicate measurements for 0.1 \(\mu M\) glycine listed in inset, yields a relative standard deviation (RSD) of 2.05\%, the detection limit \((S/N=3)\) is 0.05 \(\mu M\). A set of 11 replicate measurements for 0.5 mM glycine yielded a relative standard deviation (RSD) of 3.5\%.

Figure 9. Chromatogram of a mixture of 17 amino acids (5 \(\mu M\)) containing an internal standard of 6-aminocaproic acid. A silica-based HPLC separation column was used for separation of the mixture of AAs before the samples were entranced into FIA with the electrode proposed as the detector.

The optimized experimental condition including 25\% ratio of \(W_{Fe}/W_{Fe+CNT}\), pH 8.0, 0.6 V of the electrode potential are adopted in FIA measurements. Considering 2.5 s of response time of the electrode as the detector and the effect of flow rates for the carrier on peak height and peak area, 250 \(\mu l/min\) of flow rates was applied. The obtained results are shown in Figure 8. Besides glycine, there are many kinds of amino acids in the dialysis solution, all of which can produce the sensor response. Combined with FIA, calibration curve of glycine was obtained. Its linear range is from 0.1 to 12 \(\mu M\) with a regression equation \(I = 29.999 C_{gly} + 5.9999(R=0.9994)\), sensitivity 30.0 nA and detection limit...
sensor reaches the needs of amino acids determination in the animals. The results obtained from both two methods are consistent. It confirms that the level of various amino acids detected in hypothalamus dialysis solution of Guinea pigs were also listed in Table 3.

Table 2. Recovery of glycine in the FIA assay after silica column separation

| Spiked (μM) | Detected (μM) (n=5) | Recovery (%) |
|------------|---------------------|--------------|
| 0.10       | 0.11                | 110          |
| 0.50       | 0.48                | 96           |
| 1.00       | 1.05                | 105          |
| 5.00       | 4.91                | 98           |
| 10.00      | 10.1                | 101          |

Table 3. Comparison of Linear range, regression (R), sensitivity, and detection limit (DL) of determination of 17 amino acids using standard solution combined with FIA with Reference [11]

| Amino acids | Linear range (μM) | R | Sensitivity (nA/μM) | DL (S/N = 3) (μM) |
|-------------|-------------------|---|--------------------|-------------------|
| Ala         | 0.60-2.42         | 5-500 | 0.9985             | 0.9990           | 42.5 | 7.29 | 0.30 | 24 |
| Arg         | 0.05-6.60         | 5-300 | 0.9891             | 0.9960           | 29.4 | 5.97 | 0.03 | 587 |
| Asn         | -                 | 5-500 | 0.9894             | -                | 5.05 | -    | 695 |
| Asp         | 1.40-9.20         | 5-500 | 0.9949             | 0.9951           | 34.9 | 5.98 | 0.50 | 2693 |
| Cys         | 0.10-9.00         | 10-300 | 0.9952             | 0.9955           | 37.4 | 6.41 | 0.05 | 875 |
| Glu         | 0.10-14.0         | 5-500 | 0.9903             | 0.9901           | 28.6 | 4.90 | 0.10 | 624 |
| Gln         | -                 | 5-500 | 0.9962             | -                | 7.40 | -    | 221 |
| Gly         | 0.10-12.0         | 5-500 | 0.9994             | 0.9996           | 30.0 | 2.83 | 0.05 | 161 |
| His         | 1.90-19.8         | 5-500 | 0.9958             | 0.9961           | 70.3 | 12.07 | 0.30 | 44 |
| Iso-Leu     | 0.20-8.00         | 5-500 | 0.9974             | 0.9976           | 25.7 | 4.40 | 0.07 | 297 |
| Leu         | 0.20-12.6         | 5-300 | 0.9976             | 0.9978           | 43.7 | 7.44 | 0.08 | 231 |
| Lys         | 2.00-6.40         | 5-500 | 0.9988             | 0.9994           | 25.0 | 4.30 | 0.70 | 1890 |
| Met         | 0.20-6.00         | 5-500 | 0.9919             | 0.9929           | 52.4 | 8.99 | 0.10 | 102 |
| Phe         | 0.15-8.60         | 5-500 | 0.9987             | 0.9991           | 44.4 | 7.62 | 0.10 | 472 |
| Pro         | -                 | 5-300 | 0.9988             | -                | 3.70 | -    | 2545 |
| Ser         | 0.20-8.00         | 5-500 | 0.9911             | 0.9914           | 44.2 | 7.58 | 0.10 | 1393 |
| Thr         | 0.06-15.0         | 5-500 | 0.9952             | 0.9954           | 49.3 | 8.46 | 0.03 | 429 |
| Trp         | -                 | 5-500 | 0.9993             | -                | 6.66 | -    | 389 |
| Tyr         | 1.90-11.0         | 5-500 | 0.9998             | 0.9999           | 41.8 | 7.17 | 0.80 | 548 |
| Val         | 0.30-9.20         | 5-500 | 0.9990             | 0.9992           | 24.5 | 4.21 | 0.15 | 574 |
| GABA        | 0.10-7.50         | -    | 0.9982             | -                | 30.6 | -    | 0.05 | - |

3.7. Application

Figure 9 shows the chromatogram of a mixture of 17 amino acids (5 μM) containing an internal standard, 6-aminocaproic acid, using a silica-based separation column with the electrode detector. Furthermore, level of various amino acids detected in hypothalamus dialysis solution of Guinea pigs were listed in Table 4. The results obtained from both two methods are consistent. It confirms that the sensor reaches the needs of amino acids determination in the animals.
Table 4. Comparison of levels of 17 amino acids detected in microdialysis fluid of Guinea Pigs Hypothalamus with two methods

| Amino acids | This study (μM) | HPLC (μM) | Amino acids | This study (μM) | HPLC (μM) |
|-------------|----------------|-----------|-------------|----------------|-----------|
| Ala         | 1.83±0.15      | 1.95±0.17 | Lys         | 1.14±0.09      | 1.25±0.13 |
| Arg         | 0.81±0.06      | 1.01±0.07 | Met         | 0.13±0.01      | 0.24±0.02 |
| Asp         | 1.41±0.11      | 1.53±0.12 | Phe         | 0.66±0.05      | 0.78±0.08 |
| Cys         | 1.05±0.08      | 1.12±0.09 | Ser         | 0.52±0.04      | 0.67±0.05 |
| Glu         | 0.46±0.05      | 0.61±0.06 | Thr         | 1.69±0.14      | 1.82±0.13 |
| Gly         | 2.66±0.21      | 2.48±0.28 | Tyr         | 0.63±0.06      | 0.59±0.05 |
| His         | 1.33±0.12      | 1.55±0.14 | Val         | 1.56±0.12      | 1.44±0.10 |
| Iso-Leu     | 0.63±0.05      | 0.78±0.06 | GABA        | 0.26±0.02      | 0.23±0.02 |
| Leu         | 1.21±0.10      | 1.37±0.17 | /           | /              | /         |

4. Conclusion

The proposed electrode combined with FIA and HPLC exhibits good performances such as linear response, sensitivity. The determination of 17 amino acids in the hypothalamus microdialysis fluids of guinea pigs, illustrates that it is a powerful tool to investigate physiology and pathology mechanisms. Compared with HPLC for determination of amino acids, the biggest advantage this study is that derivatization steps can be saved, save time, save money.

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