

## Development of Electrochemical Sensor for Homocysteine Determination as a Cerebrovascular Disease Biomarker

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This work proposed the glassy carbon electrodes (GCE) based on reduced graphene oxide-TiO<sub>2</sub> (RGO-TiO<sub>2</sub>) nanocomposite to realize sensitive homocysteine (Hcy) detection. The RGO-TiO<sub>2</sub> exhibits a remarkable electrocatalytic activity to Hcy oxidation at a low potential, as indicated in cyclic voltammetric (CV) results. The RGO-TiO<sub>2</sub>-based GCE has also been found highly stable and reproducible. Furthermore, it is strongly resistant to electrode fouling. In addition to these analytical features, it requires low operation potential. Therefore the proposed RGO-TiO<sub>2</sub>-based GCE could reliably and sensitively detect Hcy, with a limit of detection (LOD) of 24 nM (S/N=3) and a desirable dynamic linearity (80 μM).

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**Keywords:** Homocysteine; Electrochemical sensor; Graphene; Biomarker; Cerebrovascular disease

### 1. INTRODUCTION

Homocysteine (Hcy), a sulfur-containing amino acid, is produced in the metabolism process of the essential amino acid methionine. The association of hyperhomocysteinemia (HHcy) with atherosclerotic disease in coronary, cerebral and peripheral blood vessels has been proved by recently reported works [1]. Although severe HHcy rarely occurs, mild Hcy increase in blood has been found to appear in 5–7% of the general population, 42% of patients with CVD and 30% with coronary artery disease (CAD) [2, 3]. A summary odds ratio (OR) for CVD was obtained as 1.9 for every increase (5 μmol/L) in total Hcy (tHcy) [4] by a meta-analysis of 27 studies, whereas an OR was obtained as 3.97 in case tHcy was higher than the 95th percentile [5]. These researches mainly focused on the large vessel atherothrombotic disease. However, Hcy has been found associated with small vessel disease

and is more likely to cause leukoaraiosis, while this has not been well confirmed [6, 7]. Besides, it has been indicated that Hcy functions as a risk factor in brain atrophy for healthy subjects [8] and increases AD risk [9]. The elevated Hcy levels have been found inversely related to the cognitive function in healthy populations by cross-sectional researches, while the results of longitudinal researches are not consistent with it [10-12]. The cognitive impairment is not necessarily ascribed to the decreased vitamin B<sub>12</sub> or folate or to the increased CVD [13]. The aforementioned results indicate Hcy is directly neurotoxic and seemingly to be dose-related.

Ordinarily, chromatographic techniques are applied to determining total Hcy in biological specimens. Since Hcy and relative compounds are highly polarized, there has been rare application of gas chromatography, thus pre-derivatization is required for volatility enhancement [14]. Despite the necessity of using derivatization for the introduction of fluorophore or chromophore groups, liquid chromatography using UV [15, 16] or fluorescence [17, 18] determination has gained frequent application. LC-MS is effective in identifying and quantifying these compounds [19].

HPLC with electrochemical determination requiring no derivatization is an effective way to detect disulphides and thiols [20]. Nevertheless, the electrooxidation reaction of homocysteine at traditional electrodes shall occur at large overpotentials [21]. A slow performance was indicated through the electrochemistry of thiol compounds at solid electrodes [22] with an overall electrochemical process, which was similar to that of biological systems, implying the interconversion of the redox couple RSH/RSSR. The electrochemical oxidation of thiols at these electrodes becomes complicated by the adsorption and the effect of electrode surface oxides [23, 24]. Chemically modified electrodes have been reported to address these drawbacks [25, 26]. In addition, pulsed electrochemical determination at Au electrodes has also been employed to clean and activate the surface of the electrode during thiols detection [27]. Carbon nanotube-based electrode [28] and platinum/poly(methyl violet) based electrode [29] have recently been proposed for electrochemical Hcy determination

In this work, a simple wet chemical approach was proposed to synthesize RGO-TiO<sub>2</sub> nanocomposite. An electrochemical RGO-TiO<sub>2</sub>-based sensor for Hcy was constructed herein. It exhibited a new electrocatalytic response towards Hcy. This work also studied the behavior of the Hcy sensor, including its LOD, linear range, anti-interference feature, and practical application.

## 2. EXPERIMENTS

### 2.1. Chemicals

Graphene oxide powder was commercially available in JCNANO, INC. Ascorbic acid, acetylcholine (Ach), poly(diallyl dimethyl ammonium chloride) (PDDA, 20 wt.%), titanium butoxide, homocysteine, uric acid (UA), glucose, 3-hydroxytyramine hydrochloride (DA) and acetic acid were commercially available in Sigma-Aldrich. Phosphate buffer solution (PBS) was obtained through adjusting the mixture of K<sub>2</sub>HPO<sub>4</sub> and KH<sub>2</sub>PO<sub>4</sub> solution (0.1 M) to a proper pH value. All other reagents

were of analytical grade and used without further purification. 18.2 M $\Omega$  cm Milli-Q water was employed for all tests.

## 2.2. Preparation of RGO-TiO<sub>2</sub> nanocomposite

Acetic acid (20 mL) was mixed with titanium butoxide (2 mL) and stirred for 0.5 h. The obtained solution was subsequently transferred into a Teflon-lined stainless steel autoclave (50 mL). After heat treatment to 150 °C in an oven for 10 h, it was left cooling to ambient temperature, and centrifugated to obtain the sediment. The obtained product was washed and heated up to 500°C to produce TiO<sub>2</sub>. The synthesis of RGO-TiO<sub>2</sub> began with the dispersion of the obtained TiO<sub>2</sub> (100 mg) into water (20 mL) under sonication, which was then added with PDDA (2 mL) under 2 h stirring. Then the mixture was centrifugated to yield the PDDA functionalized TiO<sub>2</sub>, which was then washed with water. This was followed by the dispersion of PDDA functionalized TiO<sub>2</sub> into water (20 mL). Subsequently, the PDDA functionalized TiO<sub>2</sub> was mixed with GO dispersion (2 mL, 1 mg/mL). After 60 min stirring, ammonia solution (2 mL) was introduced into the dispersion, which was then transferred into a Teflon-lined stainless steel autoclave (50 mL). After heat treatment to 120 °C for 2 h, the as-prepared mixture was centrifugated to yield the RGO-TiO<sub>2</sub> nanocomposite.

## 2.3. Characterization

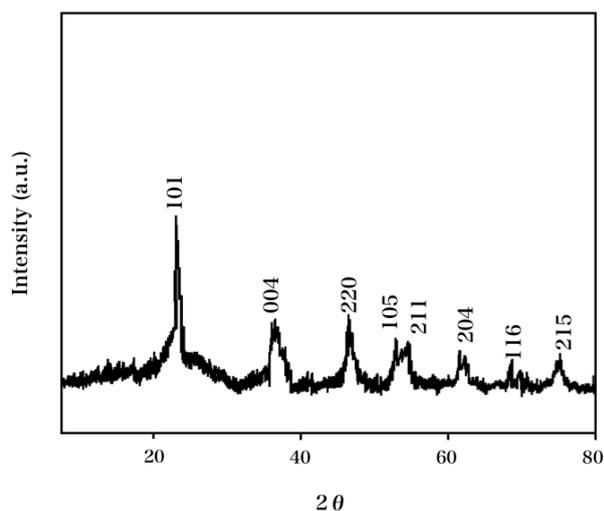
An X-ray diffractometer (D8 –Advance XRD, Bruker, Germany) with Cu K $\alpha$  radiation was used to characterize the phase structure of the prepared specimens. After alumina-water slurry polishing, the GCE was rinsed with ethanol and water for further electrochemical tests. To modify the surface of the electrode, the catalyst dispersion (5  $\mu$ L, 1 mg/mL) was dropped on the GCE, and left drying at ambient temperature. CH Instruments 660A electrochemical Workstation (CHI-660 A, CH Instruments, Texas, USA) with a triple-electrode configuration was employed for electrochemical measurements. Herein the reference and auxiliary electrode was respectively an Ag/AgCl (3M KCl) and a platinum wire. The same thin-layer radical flow cell was employed supported by a pulseless pump (Xingda Inc., Beijing, China) and an injection valve with a 5  $\mu$ l of loop (BAS) to carry out the amperometric flow injection analysis (FIA), where the working electrode was the RGO-TiO<sub>2</sub>-based electrode. All electrochemical measurements were performed at ambient temperature.

## 2.4. Determination of homocysteine in serum samples

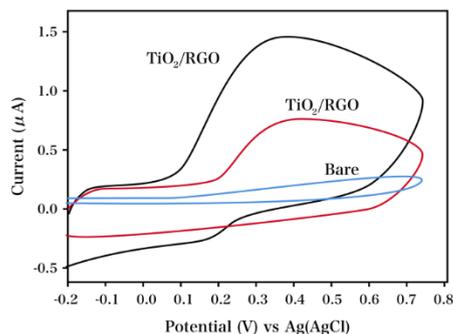
Milli-Q water (5.0 ml) was used for the reconstitution of lyophilized serum (Accutrol from Sigma). An aliquot (500  $\mu$ l) was completely dissolved, and incubated with a 10% (w/v) sodium borohydride solution (500  $\mu$ l) for 0.5 h at 50 °C. This was followed by the addition of HClO<sub>4</sub>-water solution (1250  $\mu$ l, 1:4). Then the mixed solution was stirred for homogeneization, followed by centrifugation at 3000 rpm for 10 min. Subsequently a Nylon membrane (22  $\mu$ m) was used for the filtration of the obtained solution for further electrochemical detection.

### 3. RESULTS AND DISCUSSION

The RGO-TiO<sub>2</sub> nanocomposite is characterized via XRD profiles in Fig. 1, where a range of characteristic peaks observed at 2  $\theta$  of 25.1°, 37.3°, 47.7°, 54.2°, 54.7°, 62.6°, 68.3°, 70.7° and 75.3° respectively represent the (101), (004), (200), (105), (211), (204), (116), (200), and (215) crystallographic plane of anatase. Furthermore, a broad peak overlapped with (101) plane of anatase is found to represent the TiO<sub>2</sub> incorporated-exfoliated RGO sheets.



**Figure 1.** XRD profile of RGO-TiO<sub>2</sub>.

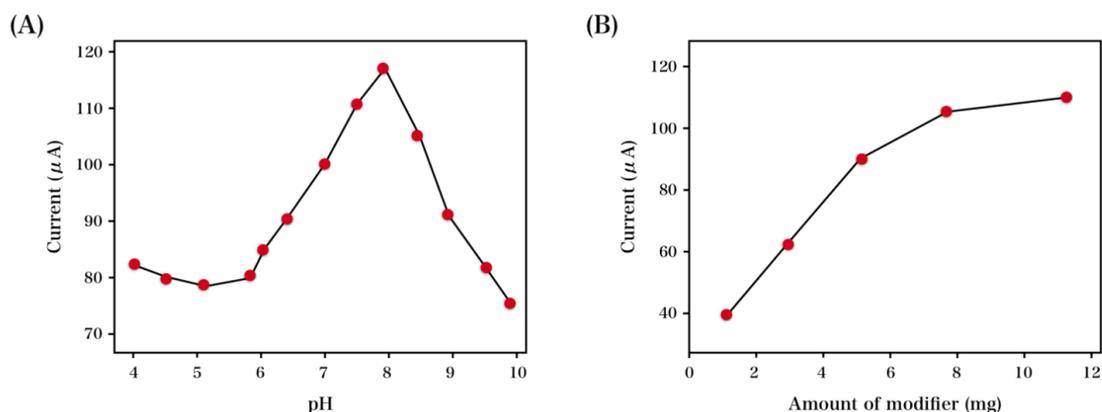


**Figure 2.** CVs of the original, TiO<sub>2</sub> and RGO-TiO<sub>2</sub>-based GCE in PBS (0.1 M, pH 8.0) with the addition of Hcy (10  $\mu$ M) with a scan rate of 50 mV/s.

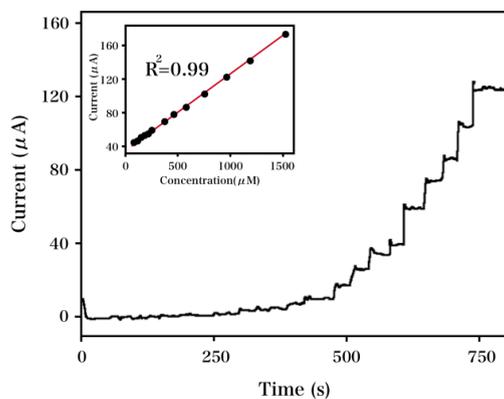
The CVs obtained at the original GCE, TiO<sub>2</sub>-based GCE and RGO-TiO<sub>2</sub>-based GCE in PBS (0.1 M, pH 8) containing Hcy (10  $\mu$ M) were put into comparison to display the electrocatalytic behavior of RGO-TiO<sub>2</sub>-based GCE. There is no obvious current response of AA at the initial GCE at the potential of  $-0.8$  —  $0.8$  V (Fig. 2), whereas the TiO<sub>2</sub>-based GCE exhibits a direct oxidation peak of AA at 0.08 V, indicating the enhancement of the electrochemical reaction rate on the surface of the electrode caused by the prepared TiO<sub>2</sub>. Moreover, the background signal observed for potential values

more positive suggests the contribution of gold oxidation processes to the current measured at these positive potentials [30]. A similar CV pattern is shown for the RGO-TiO<sub>2</sub>-based GCE except for a relatively lower oxidation peak potential of 0.02 V with higher current response, suggesting that catalyze oxidation of Hcy has been further promoted through the combination of TiO<sub>2</sub> and RGO. These results agree with those reported for other graphene based nanocomposite modified electrodes [31, 32].

The electrochemical detection has been found greatly affected by the electrolyte pH. The effect of pH value (4–10) on the RGO-TiO<sub>2</sub>-based GCE with the addition of Hcy (10 μM) is exhibited in Fig. 3A. As pH increases from 5.5 to 8, there is a gradual increase in the current response, which subsequently drops under strong alkaline circumstance. Hence the optimal pH value for the determination of Hcy is obtained as 8. In addition, the modifier amount effect on the anodic peak current of Hcy has also been studied. The *E<sub>p</sub>* value (which corresponds to the less positive peak when two peaks appeared in the voltammograms) varied with pH. As the modifier amount increases up to 7 μL, there is an obvious increase in the anodic peak current (Fig. 3B). However, the current response exhibits a slight decrease as the modifier amount is further increased, which is ascribed to the longer time for Hcy electrons to transport through the comparatively thicker RGO-TiO<sub>2</sub> film.

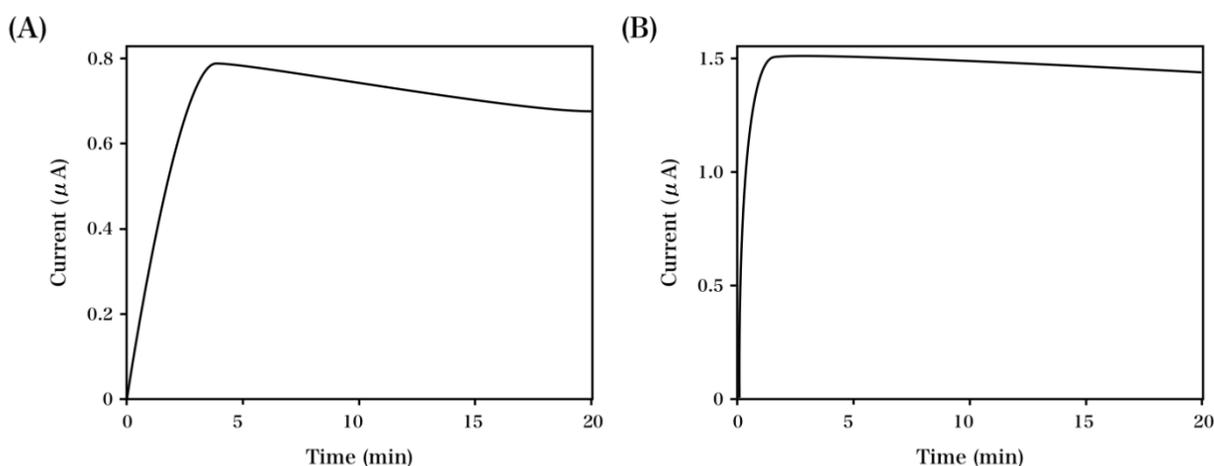


**Figure 3.** The plot of *I<sub>pa</sub>* vs. (A) PBS pH (B) modifier amount for the determination of Hcy (10 μM).



**Figure 4.** Amperometric response of the RGO-TiO<sub>2</sub>-based GCE for the successive addition of Hcy into the continuously stirred N<sub>2</sub> saturated PBS (0.1 M, pH 8.0). Inset: Calibration profile at concentrations ranging from 0.1 and 80 μM.

The characteristic amperometric response of the RGO-TiO<sub>2</sub>-based GCE is characterized after Hcy was successively added in a range of 0.1 - 80  $\mu\text{M}$  (Fig. 4). Each time after Hcy is added, there is a rapid increase in the response current, which then returns to the steady state in 3 s, indicating that the RGO-TiO<sub>2</sub> modified GCE is stable and durable for a long-time use, mainly because of the electrode could resistance of fouling and deactivation from the oxidation product of Hcy [33]. This phenomenon suggests the rapid response of the RGO-TiO<sub>2</sub>-based GCE towards Hcy. As shown in the calibration graph of the inset, a linear relationship is exhibited between the current response and the concentration of Hcy (0.1 - 80  $\mu\text{M}$ ), with a LOD of 24 nM ( $S/N = 3$ ) and a corresponding regression equation of  $I (\mu\text{A}) = 0.09114 C(\mu\text{M}) + 11.59$  ( $R_2 = 0.997$ ).



**Figure 5.** Representative amperometric I-t response of Hcy (10  $\mu\text{M}$ ) in a thin-layer flow cell with (A) the original GCE and (B) RGO-TiO<sub>2</sub>-based GCE.

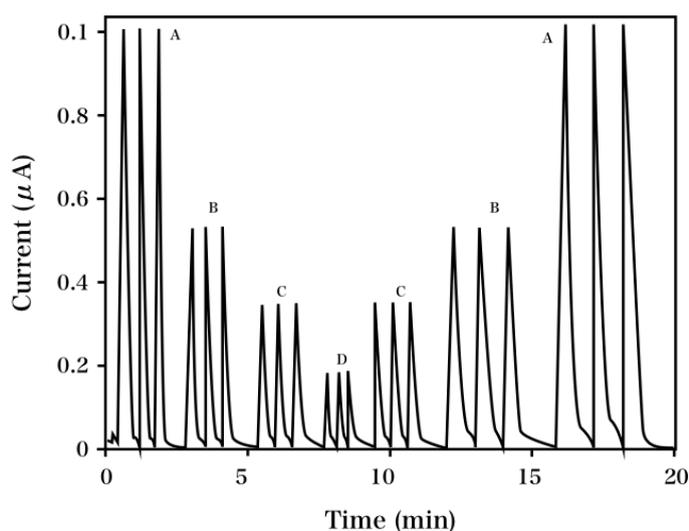
**Table 1.** A comparison of the analytical properties of electrochemical Hcy sensor with those of previously reported methods.

Technique	Linear range	Detection limit	Reference
HPLC-UV	2.5 - 10 $\mu\text{M}$	0.1 $\mu\text{M}$	[15]
HPLC-UV	0.5 - 50 $\mu\text{M}$	0.1 $\mu\text{M}$	[16]
HPLC-FL	3.9 - 62.5 $\mu\text{M}$	0.12 $\mu\text{M}$	[17]
HPLC-FL	0.05 - 25 $\mu\text{M}$	2.3 nM	[18]
HPLC-MS	1.5 - 740 $\mu\text{M}$	0.75 $\mu\text{M}$	[19]
HPLC-MS/MS	—	1.0 $\mu\text{M}$	[34]
HPLC-ED	—	0.14 $\mu\text{M}$	[35]
HPLC-ED	0.2 - 100 $\mu\text{M}$	0.1 $\mu\text{M}$	[29]
RGO-TiO <sub>2</sub> /GCE	0.1 - 80 $\mu\text{M}$	24 nM	This work

The proposed RGO-TiO<sub>2</sub>-based GCE exhibits desirable stability in Hcy detection and strong resistance to electrode fouling, as the amperometric response for Hcy at the RGO-TiO<sub>2</sub>-based GCE was compared with that of the original GCE in a continuous-flow configuration (Fig. 5). It can be seen that there is a gradual decrease in the current for the oxidization of Hcy at the original GCE as a function of time, suggesting a poor long-time stability and durability of the original GCE. This is primarily because the electrode fouling and deactivation have been induced by the oxidation product of

Hcy. The RGO-TiO<sub>2</sub>-based GCE is comparatively stable, with an extremely insignificant decrease in the current observed after successive electrode running in the continuous-flow configuration for over 60 min. Table 1 displays the comparison of the analytical features of Hcy sensor with those of previously reported HPLC techniques involving ultraviolet (UV), electrochemical (ED), mass spectrometric (MS) and fluorescence (FL) determination methods.

The reproducibility and stability of the RGO-TiO<sub>2</sub> modified GCE in Hcy detection was investigated using amperometric flow injection analysis (Fig. 6). Cysteine, which has been proved to accelerate the Hcy auto-oxidation, could also be oxidized at the electrode [36]. Well-defined peaks with a low noise were observed in proportion to the Hcy concentration. After Hcy (1.0 μM) was consecutively injected (over 50 times), no current decrease could be observed. Herein reproducible results were obtained for Hcy (1 μM), with relative standard deviation (RSD) below 4.2%.



**Figure 6.** FIA responses of Hcy with varied concentrations at the RGO-TiO<sub>2</sub>-based GCE. Carrier solution: PBS (0.10 M) at a flow rate of 0.10 mL/min.

The concentration of Hcy in human serum specimen has been employed for the evaluation of the practical behavior of the as-prepared sensor. The recovery with respect to Hcy detection ranges from 99.05 to 102.82% for the three specimens (Table 2). Hence the as-prepared Hcy sensor has the potential to be applied to the detection of Hcy concentration in real specimens.

**Table 2.** Determination of Hcy in human serum specimens.

Sample	Content (μM)	Add (μM)	Found (μM)	Recovery (%)	RSD (%)
1	1.53	10.00	11.42	99.05	4.77
2	3.62	10.00	13.57	99.63	3.52
3	12.01	10.00	22.63	102.82	1.50

#### 4. CONCLUSIONS

In this work, a simple wet chemical approach was proposed to prepare RGO-TiO<sub>2</sub> nanocomposite. The obtained RGO-TiO<sub>2</sub> was then employed for the fabrication of Hcy sensor. The RGO-TiO<sub>2</sub>-based GCE shows a more desirable electrocatalytic activity to Hcy oxidation compared with the original GCE and TiO<sub>2</sub>-based GCE. The synergistic effect between TiO<sub>2</sub> and RGO possibly explains the higher oxidation current at low oxidation potential. It has been found that the as-prepared Hcy sensor is desirably stable and selective, and possesses low LOD, wide linear range, and fast current response.

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