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Determination of glutathione and glutathione disulfide in hepatocytes by liquid chromatography with an electrode modified with functionalized carbon nanotubes

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Abstract

Glutathione (GSH) and glutathione disulfide (GSSG) are important thiols, which provide defence against oxidative stress by scavenging free radicals or causing the reduction of hydrogen peroxide. The ratio GSH/GSSG is often used as a sensitive index of oxidative stress in vivo. In this paper, a direct electrochemical method using an electrode modified with functionalized carbon nanotubes as electrochemical detector (ED) for liquid chromatography (LC) was described. The electrochemical behaviors of GSH and GSSG on this modified electrode were investigated by cyclic voltammetry and it was found that the functionalized carbon nanotubes exhibited efficiently electrocatalysis on the current responses of GSH and GSSG. In LC-ED, both of the analytes showed good and stable current responses. The detection limit of GSH was 0.2 pmol on column and that of GSSG was 1.2 pmol on column, which were low enough for the analysis of real small samples. The method was sensitive enough to detect difference in concentration of GSH and GSSG in hepatocytes from animals with and without introduction of oxidation stress by glucose or hydrogenperoxide.

Keywords: Glutathione; Glutathione disulfide; Hepatocytes; Functionalized carbon nanotubes

1. Introduction

Thiols and disulfides play an important role in metabolism and cellular homeostasis. The low molecular mass thiol, glutathione (GSH), has been found in many living cells, and serves diverse biological functions [1], such as redox regulation [2] and detoxification [3]. As an antioxidant, GSH is involved in the reaction with reactive oxygen species, H_2O_2 and other peroxides. Its oxidised form is glutathione disulfide (GSSG) [4]. Under oxidative stress the concentration of GSH will reduce, while the concentration of GSSG will increase [5]. So the ratio GSH/GSSG is often used in assessment of oxidative stress or redox status in cells.

The determination of glutathione and glutathione disulfide has been carried out with various detection techniques [6–9]. However, since GSH and GSSG are absent of spectrum groups and the derivatization is time-consuming and more difficult to avoid the oxidation of GSH, the application of fluorometric [10,11] or other photometric methods [8,12] to the determination of GSH and GSSH is limited. To eliminate the need for derivatization, Loughlin et al. [13] developed a rugged ion exchange LC/MS method to quantify GSH and GSSG simultaneously. However, high performance liquid chromatography (HPLC) combined with electrochemical detection (ED) [14,15] has many advantages in the analysis of biological compounds, such as simplicity, rapidity, high sensitivity and low cost. Several electrochemical detection methods for GSH and GSSG have been reported. The detection was often based on the reduction of GSSG on a hanging

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mercury drop electrode [16] or on the oxidation of both GSH and GSSG on an unmodified electrode [17,18]. However, with the first method, there was a problem of pollution and with the second method, GSH and GSSG were hard to be oxidized and detected on the electrode under a common potential while under a high potential, the sensitivity of electrodes always declines fast. These may be resolved by using a chemically modified electrode (CME) since the sensitivity and selectivity of HPLC-ED often can be further enhanced by this way [19,20]. When there is a kind of CME which has good electrocatalysis to the oxidation of GSH and GSSG, the electrochemical detection method for them will be much expanded.

Carbon nanotubes [21–23] are a kind of nanostructure material, which is of much interest for the high surface area, excellent electrical conductivity, significant mechanical strength and good chemical stability. These special properties of carbon nanotubes bestow them with a broad range of potential applications such as catalyst [24], templates for nanostructures [25], nanoprobes in scanning probe microscope [26] and transistors [27]. The carbon nanotubes have been successfully applied on electrode as novel CMEs [28,29] because of their ability to promote electron-transfer reactions in electrochemical reactions. However, the purified carbon nanotubes flocculate often in aqueous or common organic solutions, which limit their further manipulation and application. This would be settled after the carbon nanotubes are functionalized [30] with nitric acid and the carboxyl groups are introduced to the open ends of nanotubes. It was found that the functionalized multi-wall carbon nanotubes (MWNT-COOH) could be better dispersed in common solvents and got a very stable suspension. When the MWNT-COOH was cast on an electrode to form a MWNT-COOH CME, good electrocatalysis to analytes was often achieved. To our best knowledge, there are still no reports on the simultaneous determination of GSH and GSSG with a modified electrode, especially the MWNT-COOH modified electrode.

In this paper, a novel electrochemical detection method of GSH and GSSG was created through modifying MWNT-COOH onto a glassy carbon electrode. Coupled with HPLC, the CME was successfully applied to the simultaneous detection of GSH and GSSG. The measurement of GSH and GSSG concentration in cell is a sensitive indicator of their redox status or oxidation stress. When the hepatocytes were under oxidative stress by adding glucose and H_2O_2 [31–33], the concentration of GSH and GSSG was detected and the ratio GSH/GSSG was acquired.

2. Experimental

2.1. Chemicals and reagents

Glutathione and glutathione disulfide were of analytical grade and purchased from Sigma (St. Louis, USA). All buffer components were of analytical grade or better quality. Double-distilled deionized water was used for all solutions. Prior to use, all solutions were degassed with purified nitrogen for 20 min.

2.2. Apparatus

Electrochemical experiments were performed with a CHI832 electrochemical system (CHI Company, USA). The three-electrode system consisted of a MWNT-COOH modified electrode or a glassy carbon electrode as working electrode, a Ag/AgCl electrode as reference electrode and a gold wire electrode as counter electrode.

Liquid chromatographic experiments were conducted on a model 510 pump and a U6K injector (Waters Association, USA). The injection volume was 20 μl. The column was Luna 5u C_{18} (4.6 mm × 25 cm) (Phenomenex Company, USA) directly attached to a C_{18} precolumn (1.0 mm × 15 mm). The detector consisted of a laboratory-made thin-layer cell and a CH-I Potentiostat (Jiangsu Electrochemical Instruments Works, Jiangsu, China). The working electrode was a MWNT-COOH modified electrode or a glassy carbon electrode. The mobile phase was 0.2 mol L^{-1} phosphate buffer solution (pH 3.0, 2% acetonitrile) which was delivered at a constant flow rate of 1.0 ml min^{-1}.

2.3. Preparation of the MWNT-COOH modified electrode

MWNTs functionalized with carboxylic acid groups were prepared by refluxing multi-wall carbon nanotube in concentrated HNO_3 for 5 h. Then the suspension was filtered and the solid was carefully washed by deionized water until the pH was near 7.0. After that, the black solid was dried under the infrared lamp. On the FTIR spectra of the MWNT, the appearance of peaks at 1716 cm^{-1} and 1575 cm^{-1} corresponded to ν(C=O, –COOH) and ν(C=O, $–COO^-$) respectively, which was in accordance with the literature [23,34], indicated that –COOH and $–COO^-$ were present on the MWNTs. Dispersed by ultrasonic agitation, the MWNT-COOH suspension was then acquired by putting the powder in *N*,*N*-dimethylformamide (0.1 mg ml^{-1}).

Prior to preparation of an electrode modified with carbon nanotubes, the surface of the glassy carbon was polished with 0.3 μm alumina on a polishing micro-cloth and rinsed with deionized water. Subsequently, the electrode was ultrasonicated thoroughly with acetone, NaOH solution (1.0 mol L^{-1}), HNO_3 (1.0 mol L^{-1}) and doubly distilled water. After pretreating, 2 μl MWNT-COOH suspension was dropped onto the electrode surface (3 mm i.d.) and the solvent was evaporated under the infrared lamp.

2.4. Preparation of hepatocytes samples

Animal care was in accordance with the Guide for the Care and Use of Laboratory Animal (NIH Publication No. 86-23, 1985, Bethesda, MD). Kunming mice (20–25 g, 6

weeks old) were made diabetic by intravenous injection of alloxan (45 mg kg^{-1} body weight) in phosphate buffer (pH 7.0). Blood glucose was measured after 4 days with the intention of excluding animals with values below 20 mmol L^{-1}. Free access to food and water was allowed. Three months later, these mice were sacrificed after they were starved for 18 h and the hepatocytes were isolated. Cell viability was assessed by the trypan blue (0.4%) exclusion method.

Isolated hepatocytes (10^6 cells ml^{-1}) were incubated at 37 °C in D-Hank's buffer (pH 7.2). Oxidative stress was made by the addition of glucose or H_2O_2 to the buffer. Before analysis, the hepatocytes were centrifuged at $50 \times g$ to remove the original buffer and then suspended in 200 μl of PBS with 0.1 mmol L^{-1} EDTA. The hepatocytes suspensions were freeze-thawed three times to break the cells and then were centrifuged at $5000 \times g$ for 5 min at 4 °C. Each time 20 μl of the supernatant was injected into the column.

2.5. Statistical analysis

Results presented are means ± standard deviation of the mean. The significance of the difference between groups was established by Student's *t*-test. The *P*-values less than 0.05 were considered as statistically significant.

3. Results and discussions

3.1. Optimum detection potential

Both GSH and GSSG can be oxidized and detected on the electrode at a suitable potential. Hydrodynamic voltammetry was used to select an appropriate potential applied to HPLC-ED. The relationship between the potentials on the electrode and the relative responses of GSH and GSSG was shown in Fig. 1. Although the responses increased at higher potentials, the background and the noises increased as well. Considering both the responses and the noises, the optimum potential was +0.90 V and it was applied to the samples analysis.

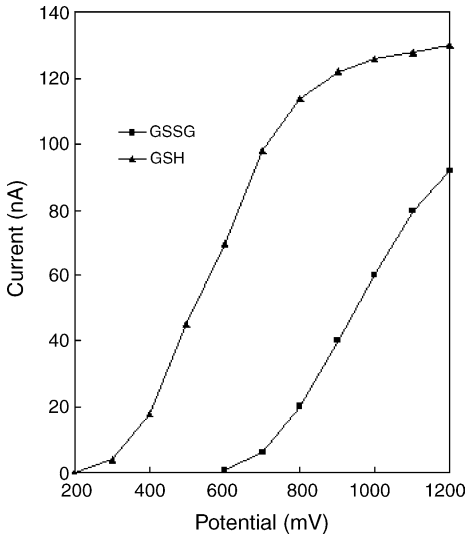

Fig. 1. Hydrodynamic voltammograms of 5×10^{-6} mol L^{-1} GSH and 1×10^{-5} mol L^{-1} GSSG at various applied potentials at HPLC-ED.

3.2. Electrocatalysis of GSH and GSSG at the MWNT-COOH modified electrode

Fig. 2 shows the cyclic voltammograms of GSH and GSSG at the unmodified glassy carbon electrode and at the MWNT-COOH modified electrode. Compared with unmodified electrode, the background current of the modified electrode was larger, which might be attributed to the increased surface charges due to nano-scale particles. After the addition of 0.2 mmol L^{-1} GSH or 1.0 mmol L^{-1} GSSG, the currents of

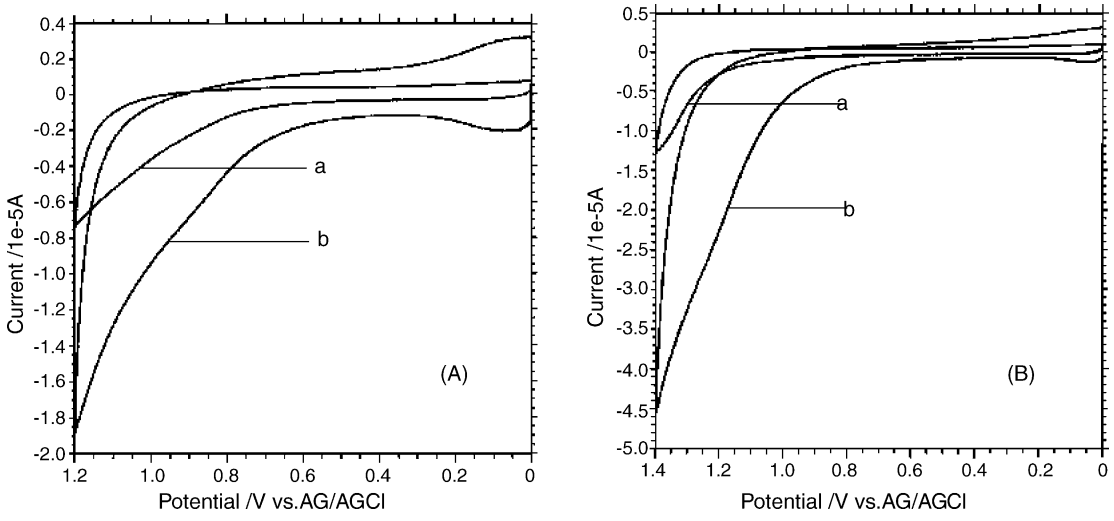

Fig. 2. Cyclic voltammograms of GSH (A) and GSSG (B) at bare glassy carbon electrode and MWNT-COOH modified electrode: (a) bare glassy carbon electrode in 0.2 mmol L^{-1} GSH or 1.0 mmol L^{-1} GSSG solution; (b) MWNT-COOH modified electrode in 0.2 mmol L^{-1} GSH or 1.0 mmol L^{-1} GSSG solution. Electrolyte: 0.20 mol L^{-1} PBS (pH 3.0); scan rate: 0.1 V/s.

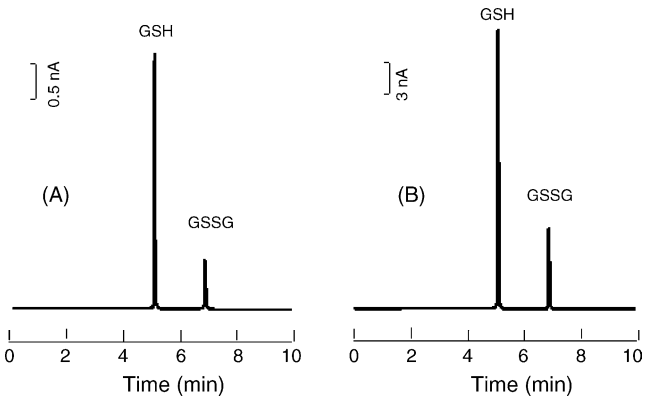

Fig. 3. Chromatograms of 1.0 μmol L^{-1} GSH and 2.0 μmol L^{-1} GSSG at bare glassy carbon electrode (A) and MWNT-COOH modified electrode (B). Column was Luna 5u C_{18} (4.6 mm × 25 cm) and directly attached to a C_{18} precolumn (1.0 mm × 15 mm) injection volume: 20 μl; mobile phase: 0.20 mol L^{-1} phosphate buffer solution, pH 3.0, 2% acetonitrile; flow rate: 1.0 ml min^{-1}; applied potential: +1.90 V.

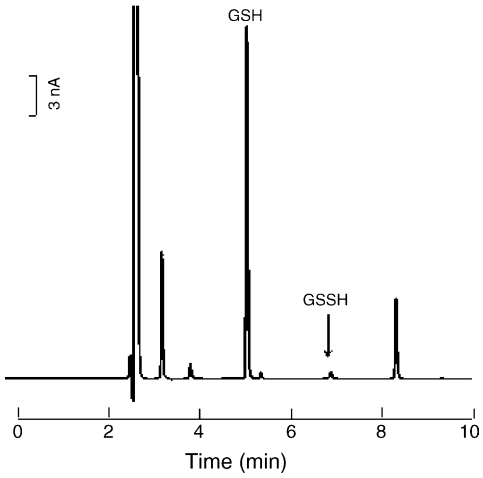

Fig. 4. Chromatogram of a hepatocytes sample. HPLC conditions were as described in Fig. 3.

their oxidation appeared and increased much more at the MWNT-COOH modified electrode than at the glassy carbon electrode.

It was also noticed that this kind of CME was more effective to increase the current of GSSG. This was observed in the chromatograms of standard solutions (Fig. 3), too. The main reason may be the high overpotential of GSSG. When there is catalysis that even causes very small decrease on the overpotential, the current response will increase much.

3.3. Linearity and detection limits

To determine the linearity of GSH and GSSG on the MWNT-COOH modified electrode in LC-ED, a series of mixed standard solutions of both analytes ranging from 1.0 nmol L^{-1} to 10 μmol L^{-1} were tested. The ranges of the linear relationships between currents and concentrations were over three orders of magnitudes and both standard curves obtained high correlations with coefficient (R^2) more than 0.998. The detection limits of these analytes at the CME were also investigated and the data were shown in Table 1.

3.4. Reproducibility and stability

The intraday assay coefficient of variation was estimated by making repetitive injection (eight times) of a standard solution containing 1.0 μmol L^{-1} GSH and 1.0 μmol L^{-1} GSSG under the same condition every 20 min. It was calculated to be 1.4% for GSH and 2.2% for GSSG. The intraday assay coefficient of variation was 2.7% for GSH and 4.3% for GSSG during 4 consecutive days. The MWNT-COOH modified electrode showed high sensitivity. Even after more than 100 injections, the sensitivity of MWNT-COOH CME didn't decrease obviously. The behaviors of the two thiols at the unmodified electrode were also investigated. It was found that the sensitivity of the unmodified electrode declined significantly after more than 100 injections. In addition, the long-term stability of the MWNT-COOH modified electrode stored at 4 °C in PBS was examined by checking its relative activity periodically. No apparent change in the current responses of both analytes was observed over 2 weeks. The results indicated that the MWNT-COOH modified electrode had good stability and reproducibility when it was used as the HPLC detector for GSH and GSSG.

3.5. The recovery of GSH and GSSG

The recovery of GSH and GSSG from cells was examined by adding them to control hepatocytes samples to about double the endogenous concentration. The recovery of GSH and GSSG was 97.5 ± 2.7% and 98.8 ± 4.6%, respectively ($n = 6$). Fig. 4 is the chromatogram of one real sample.

Table 1
The analytical data of GSH and GSSG by HPLC-ED[a] at MWNT-COOH modified electrode

Analytes	Regression equation[b]	R^2	Range	Detection limits[c] (nmol L^{-1})
GSH	$Y = 25.68X + 0.26$	0.999	20 nmol L^{-1}–20 μmol L^{-1}	10
GSSG	$Y = 3.73X - 0.03$	0.998	80 nmol L^{-1}–20 μmol L^{-1}	60

[a] HPLC-ED conditions as in Fig. 3.
[b] Y and X represent the peak current (nA) and the concentration of the analytes (μmol L^{-1}), respectively.
[c] The detection limits of the analytes were investigated using a signal-to-noise ratio of 3 (S/N = 3).

Table 2
The concentrations of GSH and GSSG in hepatocytes samples[a] ($n = 5$)

Reagents	Untreated hepatocytes			Alloxan treated hepatocytes		
	GSH (μmol L^{-1})	GSSG (nmol L^{-1})	GSH/GSSG	GSH (μmol L^{-1})	GSSG (nmol L^{-1})	GSH/GSSG
Control[b]	1.14 ± 0.05	47.3 ± 2.6	24.1	0.89 ± 0.04	47.1 ± 2.9	18.9[e]
50 mmol L^{-1} glucose	1.07 ± 0.07	69.9 ± 4.8	15.3[c]	0.79 ± 0.05	65.4 ± 4.6	12.1[d,e]
0.5‰ H_2O_2	0.94 ± 0.06	106.8 ± 7.3	8.8[c]	0.73 ± 0.05	109.6 ± 8.5	6.7[d,e]

[a] Hepatocytes were incubated at 37 °C in D-Hank's buffer for 2 h before the reagents were added into the buffer and then they were incubated for 6 h.
[b] There was no addition.
[c] $P < 0.05$, Student's t-test compared with control samples.
[d] $P < 0.01$, Student's t-test compared with control samples.
[e] $P < 0.05$, Student's t-test compared with samples untreated with alloxan.

3.6. Analytical application

Glucose of high concentration is considered to cause oxidative stress in vivo [32,33]. We prepared two types of samples to determine the effect of glucose on GSH and on GSSG in hepatocytes. The first type were common hepatocytes from healthy mice and the second type were hepatocytes from alloxan-treated mice, which had the similar symptoms with the diabetics. We determined the concentration of GSH and GSSG in the two types of hepatocytes and the oxidative stress was made by 50 mmol L^{-1} glucose or 0.5‰ H_2O_2. There was no addition in control samples. The results were shown in Table 2. It was found that without adding any reagents, the ratio GSH/GSSG in alloxan-treated hepatocytes was 18.9 and the ratio in untreated hepatocytes was 24.1. While with 50 mmol L^{-1} glucose, the ratio was 12.0 and 15.3 and with 0.5‰ H_2O_2, the ratio was 6.7 and 8.9, respectively. Table 2 demonstrates the significant ($P < 0.05$) decrease in the ratio GSH/GSSG with the treatment of glucose or H_2O_2. And the reduction of the ratio was more significant ($P < 0.01$) in alloxan-induced diabetic animals. These results indicate that glucose in high concentration does cause oxidative stress to hepatocytes just like the oxidant of H_2O_2 and in the hepatocytes treated with alloxan, this kind of oxidative stress was even greater.

In summary, a fast and sensitive electrochemical detection method for GSH and GSSG was developed. Coupled with HPLC, this method was applicable in the measurement of both GSH and GSSG in real hepatocytes samples.

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