**Selective electrochemical determination of glutathione from the
leakage of intracellular GSH contents in HeLa cells following
doxorubicin-induced cell apoptosis**

Lingzhi Zhao^{a,c,*}, Liu Zhao^b, Yanqing Miao^a, Chenxiao Zhang^{c,*}

^a *Department of pharmacy, Xi 'an Medical College, Xi 'an 710021, China*

^b *Beijing Research Center of Agricultural Standards and Testing, Beijing 100097, China*

^c *Key Laboratory of Analytical Chemistry for Life Science of Shaanxi Province, School of Chemistry and Chemical Engineering, Shaanxi Normal University, Xi'an 710062, China*

¹ Corresponding author. E-mail: oldskyhappy_zlz@163.com for Lingzhi Zhao; cxzhang@snnu.edu.cn for C.X Zhang.. Phone: +862915829583060; Fax: +86-29-81530727.

ABSTRACT

A highly selective method for the electrochemical detection of reduced glutathione (GSH) has been developed on the basis of the product of a reaction between GSH and catechol as the electron mediator on single-walled carbon nanotubes modified glassy carbon electrode. A polymerization-adduct ECE mechanism at SWNTs /GCE was proposed. The oxidation peak potential was -0.160 V (vs. Ag /AgCl, sat. KCl) and the oxidation peak current showed a dependence relationship with GSH concentration in the range of 1.0 ~500.0 μ M with a detection limit of 0.5 μ M GSH. The developed method can discriminate GSH from other thiol species such as L-cysteine (Cys) and cysteamine hydrochloride (Cym) within their certain concentration ranges. The as-established electrochemical method has been successfully applied for the determination of GSH from the leakage of intracellular GSH contents in HeLa cells following doxorubicin-induced cell apoptosis.

Keywords: Glutathione (GSH), Biothiols, Electrochemical detection, Catechol derivatives, Single-walled carbon nanotubes (SWNTs)

1. Introduction

Glutathione (GSH), one of endogenous thiol compounds, is the main intracellular tripeptide that serves diverse cellular functions, such as maintenance of intracellular redox and amino acid homeostasis, gene regulation, and intracellular signal transduction. GSH can be oxidized to GSSG, with a disulfide bond formation, which is a key posttranslational modification with implications for structure, function and stability of numerous proteins. When thiol-disulfide bond homeostasis is disrupted in relation to the altered GSH concentration in biological fluids and inside the cells, cells

go to great lengths to regulate this homeostasis. Therefore, GSH is a valuable marker for several human diseases, such as occlusive vascular, leukemia, diabetes, and some kinds of cancers [1-5]. Many analytical methods have been developed for the determination of GSH in biological and pharmaceutical samples, such as, spectrophotometry [6-9], fluorescent [10-12], resonance elastic light scattering (RELS) [13-15], high performance liquid chromatography [16], capillary electrophoresis [17-18] and electrochemical methods [19-44]. Among these methods, the electrochemical methods based on direct detection or electrocatalytic approach have attracted greater attention due to their simplicity, sensitivity, lower cost, and so forth.

Extensive efforts have been devoted to developing novel electrochemical methods for the detection of GSH with high sensitivity, reproducibility, simplicity and reusability. These efforts included new electrode materials and appropriate electron mediators. The development of new electrode materials included employing a variety of nanomaterials, such as, sol-gel-derived ceramic-carbon nanotube nanocomposite electrodes reported by Gong et al [22], a nickel hexacyanoferrate film modified Pt ultramicroelectrode by introducing cetyltrimethylammonium bromide and Au nanoparticles reported by Lu et al [28], and Pt–NiCo Nanostructures modified glass carbon electrodes reported by Tian et al and so forth [20]. These nanomaterials performed high electrocatalytic activity and stability for the oxidation of GSH. What is more, combining with nanomaterials, appropriate electron-transfer mediators were synthesized for the in vitro assay of GSH. Since McCarley [19] developed a simple electrochemical approach for detecting the biothiols with catechol derivatives as the mediators and proposed reaction pathway for the addition of thiols to the catechol analogues derivative via the ECE mechanism, catechol analogues were extensively employed as the mediators for the in vitro assay of thiol compounds, such as Cys

(cysteine), Hcy (homocysteine) and GSH. Karimi-Maleh et al [25] reported a highly sensitive electrochemical sensor based on a FePt / CNTs nanocomposite with a novel amide ligand (9,10-dihydro-9,10-ethanoanthracene-11,12-dicarboximido)-4-ethylbenzene-1,2-diol (DED) as a mediator for the determination of GSH. DED as one of catechol analogues with great conjugated structure made it easier immobilized on the surface of electrodes or nanomaterials. Raoof et al [30] reported carbon paste electrode modified with 4, 5-bis (4-chloroanilino)-1,2- benzendiol (BCB) and ZnO /CNTs for the voltammetric determination of GSH in the presence of NADH and folic acid. However, to our best knowledge, these methods based on nanomaterials and reported mediators could not discriminate GSH from other thiol species such as Cys and Hcy.

Electrochemical sensing of GSH with catechol analogues as the mediators is based on the formation of the catechol-thiol adducts, which are the product of the corresponding quinones (the oxidation products of catechol analogues) undergoing nucleophilic substitution by the sulfhydryl group. Owing to their high structural similarity and containing a sulfhydryl group, biothiols, such as GSH, Cys and Hcy are quite difficult to be discriminated by simply using catechol analogues as the mediators. Therefore, most efforts should be devoted to search for new mediators and novel nanomaterials to improve the sensitivity and selectivity of the electrochemical sensors for the determination of GSH.

The aim of this work is to develop a sensitive and selective electrochemical method for the determination of GSH and to discriminate three kinds of biothiols. In this work, at first, we investigated the voltammetric behavior of catechol at the surface of different modified electrodes. Secondly, electrochemical behaviors of three kinds of catechol analogues (catechol, dopamine, 4-nitrocatechol) employed as the

mediators for GSH and other thiols were studied. Thirdly, based on obtained data, we developed an electrocatalytic method for selective electrochemical sensing of GSH on the basis of the product of a reaction between GSH and catechol on single-walled carbon nanotubes (SWNTs) modified glassy carbon electrode (GCE). As the model application for real samples, this method can detect GSH from the leakage of intracellular GSH contents in HeLa cells following doxorubicin-caused cell apoptosis. Compared to the reported electrochemical methods with other catechol analogues as the mediators, the main advantage of the as-established method is its selectivity for the detection of GSH from other thiol species, which often coexists with GSH in biological samples. With the striking analytical performance, the as-established system provides a methodology for sensing GSH with high selectivity, which is very significant to open a new pathway for the applications in physiological and pathological studies, as well as clinical diagnosis.

2. Experimental

2.1. Apparatus and reagents

Glutathione (GSH) and homocysteine (Hcy) were purchased from Sigma-Aldrich. Cysteamine hydrochloride (Cym), L-Cysteine (Cys), L-Dithiothreitol (DTT), 11-Mercaptoundecanoic acid (MUA), 6-Mercapto-1-hexanol (MCH), and other amino acids were obtained from Aladdin Company. Dulbecco's modified Eagle Medium (DMEM, high glucose) was obtained from Gibco Invitrogen, Carlsbad, CA. Fetal bovine serum (FBS, South American Origin) was obtained from Hyclone. Single-walled carbon nanotubes (with an average diameter less than 2 nm, SWNTs), multi-walled carbon nanotubes (with an average diameter 8-15 nm, MWNTs) and reduced graphene (rGO) were obtained from Chengdu Organic Chemicals Co., Ltd., Chinese Academy of Sciences (Chengdu, China). Phosphate-buffered saline (PBS,

pH 7.4) consisting of 136.7mM NaCl, 2.7mM KCl, 0.087M Na₂HPO₄, and 0.014M KH₂PO₄ was prepared. Aqueous solutions of GSH, cysteine and homocysteine were freshly prepared with PBS. All reagents were at least analytical grade and used as received. Aqueous solutions were prepared with Milli-Q water.

2.2. Fabrication of modified electrode

Glassy Carbon (GC) electrodes (2.0 mm diameter) were polished with 0.3 and 0.05 μm alumina slurry on a polishing cloth, cleaned under bath sonication for 5 min in acetone and distilled water, respectively, and thoroughly rinsed with Milli-Q water. 1 mg SWNTs (or MWNTs) was dispersed into 1 mL of N,N-dimethylformamide (DMF) to obtain a homogeneous suspension under sonication. 3 μL of the prepared suspension was drop-coated onto the surface of cleaned GC electrodes to obtain the SWNTs-modified, or MWNTs-modified electrodes (SWNTs/GCE, MWNTs/GCE).

2.3. Apparatus and Measurements.

Cyclic voltammetry (CV) was performed on an electrochemical analyzer (CHI 660E, CH Instruments) with the SWNT-modified GCE as working electrode and a Pt wire as counter electrode and Ag/AgCl electrode (KCl-saturated) as reference electrode. All potentials were referred with respect to this reference electrode.

Field transmission electron microscope (TEM) were recorded with the Tecnai G2 F20 (FEI Company, USA) operating at 200 kV accelerated voltage. Fourier transform infrared spectrum (FTIR) was measured by the Tensor 27 FTIR system (Bruker, Germany).

2.4 Cell culture and monitoring extracellular GSH

The human cervical carcinoma cells (HeLa cells, Peking Union Medical College, Tsinghua University) were cultured in DMEM medium supplemented with 10% fetal bovine serum, penicillin (100 $\mu\text{g/mL}$), and streptomycin (100 $\mu\text{g/mL}$) in an humid

incubator (5% CO₂, 37 °C). After incubation for some time (typically, 2 days), the cells were trypsinized with 0.25% (v/v) trypsin solution dissolved in phosphate buffer (pH 7.4) containing 0.02% EDTA when the adherent cells approached confluence. Cell number was determined using a traditional counting chamber (Thoma). A Leica inverted microscope (DMI 6000) equipped with a digital monochrome camera (Leica DFC 350 FX) with software provided by Leica (LAS AF) was used to visualize the cells. Prior to the electrochemical measurements, the medium was changed to 1 mL PBS containing 10 mM glucose, and HeLa cells were cultured for the different time for subsequent detection.

3. Results and discussion

3.1 Cyclic voltammetric response of GSH and catechol at SWNTs modified GCE

In this work, since catechol was employed as the electron-transfer mediator for the electrochemical determination of Cys, Hcy and GSH, initially, the voltammetric behavior of catechol in the absence of GSH was examined at three kinds of carbon nanomaterials-modified GCE containing SWNTs, MWNTs and reduced graphene (rGO), although these nanomaterials have been reported to have good electrocatalytic activity on the electrochemical oxidation-reduction behavior of catechol in different cases [47-49]. As displayed in Fig. 1A, curve a, at bare GCE, the anodic and cathodic peak potential of catechol located at about 0.250 V and 0.100 V, respectively. The peak potential separation (ΔE_p) was 0.150 V, manifesting the characteristics of quasi-reversible redox process. In Fig. 1A, curve b (at rGO/GCE), a quasi-reversible redox behavior was observed and the peak current were increased. As seen in Fig. 1A curve c (at MWNTs /GCE) and curve d (at SWNTs /GCE), the anodic peak potential shifted negatively from 0.250 V to 0.180 V and the cathodic peak potential shifted positively from 0.100 V to 0.180 V with the ΔE_p of zero, and the peak currents were

greatly increased with a nearly unity of the peak current ratio (I_{pC}/I_{pA}). These can be attributed to the fact that the adsorption of catechol occurred on the surface of these two kinds of modified GCE. On the whole, the peak current magnitude at SWNTs/GCE was the highest at bare GCE and carbon nanomaterials modified GCE. These observations denote efficient electrocatalytic function of SWNTs/GCE for the electrochemical oxidation-reduction behavior of catechol. Therefore, SWNTs was chosen as modified material for subsequent GSH detection in this work.

Fig. 1B shows the cyclic voltammograms of GSH in PBS (pH 7.4) at SWNTs/GCE in the absence of catechol. The oxidation peak potential located at 0.580 V and no corresponding reduction peak appeared for 1.0 mM (curve b) or 10.0 mM GSH (curve c). This indicates that the electrochemical process of GSH was irreversible at SWNTs/GCE in the absence of the mediator catechol.

Figure 1C shows the cyclic voltammograms of GSH at bare GCE in the presence of catechol. Catechol exhibited a pair of quasi-reversible redox peaks with the E_{pa} at 0.260 V and the E_{pc} at 0.100 V at bare GCE in the absence of GSH (curve a). On the addition of GSH into the catechol solution (curve b), the oxidation peak current at 0.260 V was greatly increased, while the reduction peak current at 0.100 V was decreased. This indicates that the excellent electro-catalytic oxidation performance of catechol toward GSH oxidation at bare GCE occurred.

Fig. 1D shows the cyclic voltammograms of GSH at SWNTs/GCE in the presence (curve b) of the mediator catechol. In the absence of catechol, a pair of reversible adsorption waves of catechol were observed at 0.200 V. On the addition of GSH into catechol solution, a pair of well defined reversible adsorption waves of catechol appeared at -0.160 V and the peak currents greatly increased, while the peak currents at 0.200 V greatly decreased. More importantly, the oxidation peak current at

-0.160 V was found to be proportional to GSH concentration. This would eventually constitute a straightforward basis for effective electrochemical sensing of GSH. We ascribed this pair of new redox peaks at -0.160 V to the electro-oxidation of the catechol-thiol adduct which was formed from the electrochemically initiated reaction pathway, the mechanism is detailed in Scheme 1 and will be discussed later.

Moreover, the cyclic voltammetric behavior of different concentration of GSH in PBS (pH 7.4) at MWNTs/GCE in the presence of the mediator 50 μ M catechol was investigated (see **Fig. S2**). The results demonstrated that a pair of new redox peaks also located at -0.160 V. And, the peak current of the catechol-GSH adduct observed at SWNTs /GCE is higher than that at MWNTs/GCE. Therefore, SWNTs was chosen as modified material for GSH detection in this work.

3.2 Choice of the mediators

As described before, catechol analogues have been extensively reported as the electron mediators for the in vitro assay of thiol compounds such as Cys, Hcy and GSH. There have been good reasoning and validating in the literatures for the possible mechanisms towards the reaction between electro-oxidized catechol analogues (o-benzoquinone) and thiols. Based on the previously reported pathway and the data obtained in our work, herein, we proposed two pathways and further explained them. The first one is electro-catalytic mechanism as shown in Scheme 1A. The second one is polymerization-adduct ECE mechanism at SWNTs /GCE as shown in Scheme 1B. In order to understand clearly the pathways proposed in this work, we briefly describe the pathways previously reported. Nematollahi et al.[33] and Compton et al [39] proposed electro-catalytic mechanism involving reduction of electrochemically oxidized catechol analogues by Cys or Hcy. McCarley et al [19] proposed the adduct ECE mechanism at GCE, involving the 1,4-Michael addition reaction for the addition

of GSH to the catechol analogues at bare GCE: First, catechol analogues A is electrochemically oxidized to quinone B, an electron-deficient center which undergoes nucleophilic attack by the thiol species to produce the catechol-thiol adduct C, which is catechol-(monothiol). The oxidation of C yielded quinone D, which can further react with the thiol to generate catechol- (bisthiol) adduct E. In this work, we supposed an alternative pathway, that is, polymerization-adduct ECE mechanism at SWNTs /GCE, as shown in Scheme 1 B [45-46] since the potential range of CVs was from -0.400 V to 0.600 V and the peak potential observed at SWNTs /GCE negatively shifted from 0.200 V at bare GCE to -0.160 V at SWNTs. In order to support our supposed pathway, TEM was firstly used to investigate the morphology for SWNTs /GCE and SWNTs /GCE after electrochemical treatment with the catechol, respectively. The results are showed in Fig.2. These TEM images showed an obvious difference between the pristine SWNTs (A, B) and the SWNTs which was treated electrochemically in catechol solution (C, D, E). In details, the tubular structure with quite smooth surface was observed from pristine SWNTs. The SWNTs treated electrochemically in catechol solution exhibited the forming of nanosheets with the flower structure. This demonstrated the formation of polycatechol nanosheets on SWNTs /GCE [46]. The formation of polycatechol was also supported by the obtained the FTIR spectra (see Fig.S1). The intense absorption peaks at 1614 cm^{-1} and 3400 cm^{-1} are observed, ascribed to the stretching vibration of aromatic rings and catechol-OH groups for SWNTs after electrochemical treatment with the catechol, respectively. The characteristic peaks can also be discerned in the pure catechol sample. These indicate that there was in-situ polycatechol formation on SWNTs /GCE.

In order to establish a highly selective electrochemical method for the detection

of GSH with catechol analogues as the mediators, based on our proposed pathway, three catechol analogues mediators including 4-nitrocatechol, dopamine and catechol were examined. From the view molecule structure (Fig.3 A), compared with catechol, 4-nitrocatechol also contains a nitro group in meta benzenediol, which is an electron withdrawing group and decreases the electron density of benzene ring, while dopamine also contains an amino ethyl group, which is an electron-donating group and increases the electron density of the benzene ring. Thus, these three catechol analogues must have different activity of the nucleophilic substitution by the thiol species. Herein, the electrochemical behaviors of these catechol analogues were studied as the electron mediators of GSH at SWNTs/GCE.

Figure 3 (B) shows the cyclic voltammograms in PBS (pH 7.4) containing 100 μ M 4-nitrocatechol and different concentration of GSH at SWNTs/GCE. In the absence of GSH, a pair of redox peaks of 4-nitrocatechol appeared at about 0.00V. In the presence of 100 μ M GSH, a new oxidation peak at about 0.400V was observed without the corresponding reduction peak. Moreover, the oxidation peak currents at 0.00V and 0.400V slightly increase with increasing the concentration of GSH. Thus, 4-Nitrocatechol was not chosen as the electron mediator in this work.

Figure 3(C) shows the cyclic voltammograms in PBS (pH 7.4) containing 100 μ M dopamine and different concentration of GSH at SWNTs/GCE. In the absence of GSH, two pair of redox peaks of dopamine appeared at 0.200V and -0.260V. On the addition of GSH into the solution, a new oxidation peak appeared at -0.120 V and the current response for 100 μ M GSH was 6 μ A. It was found that this peak current was directly proportional to GSH concentration. This suggests that dopamine could be used as the electron mediator in tested system. However, we found that when dopamine was used for the detection of GSH in the presence of Cys and Hcy, the

detection of GSH was strongly interfered by Cys and Hcy since the nearly same current responses were observed at the same concentration of GSH, Cys and Hcy (Data not shown). These indicate that GSH can not be selectively detected with dopamine as the electron mediator.

Figure 3(D) shows the cyclic voltammograms in PBS (pH 7.4) containing 50 μM catechol and different concentration of GSH at SWNTs/GCE. In the absence of GSH, a pair of redox peaks of catechol located at about 0.200 V. On the addition of GSH into the solution, a pair of new redox peaks appeared at -0.160 V and the peak current for 100 μM GSH was 260 μA . With the increase of the concentration of GSH, the oxidation peak at -0.160 V decreased and a slight increased shoulder peak appeared at -0.049 V. The voltammetric behavior of other biothiols including Hcy, Cys and Cym was also checked at the same experiment condition. The results revealed that the addition of the other biothiols did not yield a pair of new redox peaks at -0.160 V and -0.049 V except Hcy (the corresponding data shown in Fig.5). Since the peak current of redox peaks at -0.160 V showed the excellent linear response toward certain concentration range of GSH, catechol was chosen as the mediator in this work and subsequently exploited to constitute an electrochemical detection strategy for effective electrochemical sensing of GSH.

3.3 Effect of pH

In order to obtain a high sensitivity, the effect of pH on electrochemical behaviors was examined at SWNTs/GCE in different pH buffer with typical pH values (pH 1.0, pH 5.0, pH 7.4, pH 10.0, pH 12.0) containing 50 μM catechol in the absence/presence of 100 μM GSH. In the absence of GSH (Fig. 4A), the peak currents increased with decreasing pH in the pH range of 1.0 to 12.0, and the largest current response appeared at pH 1.0. This indicates that, acidic condition was in favor of the

electrochemical processes in the tested system. Additionally, the redox peak potentials negatively shift gradually with a increase of pH value. This suggests that H^+ participated in this processes. Two electrons and two protons transfer processes of catechol has been demonstrated in Scheme 1B.

In the presence of 100 μ M GSH (Fig. 4,B), the largest current response appeared at pH 7.4. This could be reasonably explained by the polymerization-adduct ECE mechanism proposed in this work. In the reaction processes involved, catechol is first electrochemically oxidized to O-quinone, and then this electron-deficient center undergoes nucleophilic attack by GSH, results in producing the catechol-GSH adduct. The sulfhydryl group ($-SH$) of GSH attacks O-quinone based on its strong nucleophilicity. Alkaline condition could cause the sulfhydryl group to lose its proton, to form $-S^-$. It is well known that the nucleophilicity of $-S^-$ is stronger than that of $-SH$. Therefore, alkaline condition is in favor of the formation of the catechol-GSH adduct. On the other hands, acidic condition is in favor of the electrochemical oxidation of catechol, which is supported by the obtained data (Fig. 4A)). The neutral condition pH 7.4 could balance both requirements, and should be best. Since a higher sensitivity was obtained in the neutral condition pH 7.4, therefore, 0.1 M PBS (pH 7.4) was selected as the pH buffer in this work.

3.4 Interference study

To evaluate the selectivity of our method for the determination of GSH, the voltammetric behavior of other thiol species as interference compounds due to their high structural similarity with GSH was investigated, including Cys (A), Hcy (B), MUA (C), Cym (D), MCH (E), DTT (F), as depicted in Fig.5. As seen, the addition of other thiol species did not yield a pair of new redox peaks at -0.160 V except Hcy. However, the oxidation current response for 100 μ M Hcy at - 0.160 V was about 100

μA , which is smaller than the response of $100\ \mu\text{M}$ GSH (about $260\ \mu\text{A}$) and there was not a increased shoulder peak at $-0.049\ \text{V}$ as similar to GSH. These results substantially demonstrated that the proposed method had a high selectivity against other thiol species except Hcy. Moreover, the phenomenon that a pair of new redox peaks appeared at $-0.160\ \text{V}$ for Hcy demonstrated that the ECE mechanism for Hcy in the presence of the mediator catechol at SWNTs /GCE, which was different from the reported results at bare GCE [39].

Since the aim of the proposed GSH detection system was to be applied to electrochemically monitoring changes of GSH in cell culture medium following doxorubicin-induced cells apoptosis, thus, it was necessary to validate whether there were interferences on this detecting system from the reagent doxorubicin, which was employed to construct "Apoptosis" pathological models. Additionally, the physiological substances and inorganic saline, such as K^+ , Na^+ , Ca^{2+} , Mg^{2+} , uric acid, glucose, lactate, ATP, hypoxanthine, also involved in cell culture medium and inside cells which were likely to interfere with GSH detection. Therefore, these compounds should be tested. Adding them into the detection system, we observed whether they induced any evident response in this system, that is, under normal metabolic or pathological conditions, the concentrations of the above-mentioned physiological substances were used to maintain intracellular dynamic balance state and strictly subject to cellular membrane regulation with response to external stimuli. The results showed that these ten substances as interferences (containing $15\ \mu\text{g/mL}$ doxorubicin, $5\ \text{mM}$ of K^+ , Na^+ , Ca^{2+} , and glucose, $2\ \text{mM}$ of Mg^{2+} uric acid, lactate, ATP, and hypoxanthine) had no observable current response changes (no more than $\pm 5\%$ interference) for the determination of GSH ($10\ \mu\text{M}$). This indicates that the developed method has a relatively high selectivity for GSH detection within a certain range of

their concentration related to physiological changes.

3.5 Analytical performance

Under the optimum conditions, analytical performance of the developed method was examined. Fig.6A and 6B displayed typical voltammetric response toward the successive additions of GSH in the presence of 50 μM catechol. In the range concentration of GSH from 5 μM to 100 μM (Fig.6A), a well-defined increase in the oxidation peak current at about -0.160 V was observed with successive increments of GSH, and the linear equation was $I (\mu\text{A}) = 3.094 C_{\text{GSH}} (\mu\text{M}) + 29.96$ ($R = 0.984$). However, in the range concentration of GSH from 100 μM to 500 μM (Fig.6B), a decreased peak current located at -0.160 V (I_1) and a slight increased shoulder peak (I_2) appeared at -0.049V. The ratio value of I_1 / I_2 decreased with the increase of GSH concentration, and the linear equation was $I_1/I_2 = -0.00218C_{\text{GSH}} (\mu\text{M}) + 1.873$ ($R=0.984$). Although the sensitivity (slope) is low, it maybe potentially applied to assay a high concentration of GSH.

We also found that there was the dependency of the peak current of GSH on the ratio of $C_{\text{catechol}} / C_{\text{GSH}}$. Fig. 6C and Fig. 6D displayed the typical voltammetric response toward the successive additions of GSH in the presence of the mediator 75 μM and 10 μM catechol, respectively. The linear equations were $I (\mu\text{A}) = 3.17C_{\text{GSH}} (\mu\text{M}) - 3.36$ ($R = 0.991$) for 75 μM catechol and $I (\mu\text{A}) = 3.81C_{\text{GSH}} (\mu\text{M}) + 3.73$ ($R = 0.982$) for 10 μM catechol, respectively. These indicate that the established method has multiple linear calibration ranges, which is depended on the ratio of $C_{\text{GSH}}/C_{\text{catechol}}$. A wide linear range for GSH detection is achieved by just only changing the concentration of the mediator catechol. The detection limit of the developed method for the determination of GSH was calculated to be 0.5 μM (signal to noise ratio = 3) in the presence of 50 μM catechol.

Table 1 presented comparisons of the results for GSH detection obtained from our established method and those from electrochemical methods recently reported. The detection limit for GSH in this work was comparable or even better than that from different electrochemical methods based on different materials modified electrodes. Although our established method has not the highest sensitivity and the lowest detection limit among these methods, it exhibits the lowest oxidation potential and multiple linear ranges for GSH. Such a low oxidation potential benefits GSH detection and prevents from interferences from other thiol species, doxorubicin, physiological substances and inorganic salines tested in this work.

3.6 Determination of GSH from the leakage of intracellular GSH contents in HeLa cells following doxorubicin-induced cell apoptosis

In order to evaluate the reliability of the developed method for the determination of GSH in real samples, the dynamic changes of GSH in cell culture medium following doxorubicin-induced cells apoptosis were monitored. Before GSH measurements, it was necessary to employ appropriate dosage of doxorubicin to constitute cells apoptosis condition. Herein, doxorubicin (the final concentration: 15 $\mu\text{g/mL}$) was added into 0.1 M PBS containing 10mM glucose incubating HeLa cells. Fig. 7, Picture B showed the normal status of HeLa cells without doxorubicin treatment, while **Fig. 7**, Picture C showed the pathological state of HeLa cells on the glass substrate incubating 15 $\mu\text{g/mL}$ doxorubicin after 1 h. The cell body shrinkage, round and detachment from the substrate can be observed. These indicate that programmed cell death for most of cells had been launched with loss of cellular three-dimensional structures and increase of cell permeabilization at such a dosage of doxorubicin after 1 h incubation time. These pictures illustrated that 15 $\mu\text{g/mL}$ doxorubicin was enough to induce HeLa cells apoptosis. From above observation, the

morphological changes can be studied following the apoptosis induced by anti-cancer drug doxorubicin. Therefore, the dynamic change of GSH concentration in cell culture medium incubating HeLa cells may be recorded via this as-established electrochemical method.

It is well known that real samples are too complex, so that the concentration of analyte in real samples is difficultly estimated. In order to make a decision for selection of different type of calibration equations, the changes of GSH concentration from the leakage of intracellular GSH contents in HeLa cells were studied in the presence of the mediator 10 μM , 50 μM , 75 μM catechol, respectively. The results repeatedly showed that the current responses for GSH from the leakage of intracellular GSH contents in HeLa cells in the normal state and following doxorubicin-induced cell apoptosis were observed in the presence of 10 μM or 50 μM catechol, not observed in the presence of 75 μM catechol. This suggests that our method is suitable to detect GSH from the leakage of intracellular GSH contents in HeLa cells in the presence of 10 μM or 50 μM catechol. A typical CVs for the leakage of intracellular GSH contents in HeLa cells in the presence of 50 μM catechol is displayed in Fig. 7A. The basal level of extracellular GSH under the normal metabolism condition in almost 1 h was determined to be $1.1 \pm 0.30 \mu\text{M}$ ($n=3$) (curve c). When 15 $\mu\text{g/mL}$ doxorubicin was added into PBS including 10mM glucose incubating HeLa cells after 1 h, it was observed that the current response had a observable increase at -0.160 V (curve d), which was ascribed to the response of GSH. Based on the calibration equation $I (\mu\text{A}) = 3.094 C_{\text{GSH}} (\mu\text{M}) + 29.96$, the level of extracellular GSH was calculated to be $7.5 \pm 0.46 \mu\text{M}$ ($n=3$) at the time point of 60 min.

The increase of extracellular GSH concentration for HeLa cells following

doxorubicin-induced cell apoptosis was attributed to the increase in the cell membrane permeability which has been confirmed in our previous reports for MDCK cells following FCCP-induced cell apoptosis [50]. Cell membrane permeability can directly reflect the degree of cell death as a parameter. Under the normal metabolism condition, since the cell membrane is intact, the transmembrane transport of intracellular substances is strictly maintained by bioactive proteins and ion channels on the surface of cell membrane. When HeLa cells were treated 15 $\mu\text{g/mL}$ doxorubicin, programmed cell death for most of cells has been launched and the increase of cell permeabilization resulted in the increase of GSH concentration outside HeLa cells, which was leaked out from cytoplasm. The results for the change of GSH concentration in PBS incubating HeLa cells following doxorubicin-induced cells apoptosis substantially demonstrate that the developed analytical method establishes a reliable approach for the determination of GSH in the biological and cellular system.

4. Conclusion

This work is the first demonstration of different electrochemical behavior of GSH and other thiol compounds using catechol derivatives with different substituents as the mediators. The results show that the best electron mediator for GSH detection is catechol. On this basis, the proposed method with the combination of SWNTs and catechol demonstrated the superior analytical sensing performance for GSH detection. The principle is based on the catechol-GSH adduct formation, which have produced the greater electrochemical response than GSH itself and the oxidation peak potential shifted negatively from 0.600 V for the electro-oxidation of GSH to -0.160 V for the catechol-GSH adduct, where other thiol species such as Cys and Cym and other biological substances almost have no interference on GSH detection within a certain

range of their concentration. The applicability of the developed method is demonstrated for successfully detecting GSH from the leakage of intracellular GSH contents in HeLa cells following doxorubicin-induced cell apoptosis. This work provides a methodology for sensing GSH with high selectivity. The developed method could be potentially useful in physiological and pathological studies for GSH.

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Fig.1 (A) Cyclic voltammograms (CVs) of 1.0 mM catechol at bare GC electrode (curve a), 50 μ M catechol obtained at reduced graphene modified GC electrode (curve b), at MWNTs /GCE (curve c), and at SWNTs/GCE (curve d). (B). CVs for 100 μ M (curve a), 1.0 mM (curve b), 10.0 mM (curve c) GSH oxidation at SWNTs /GCE in the absence of the mediator catechol. (C). CVs of 1.0 mM catechol at bare GC electrode in the absence (curve a) and the presence of 1.0 mM GSH (curve b). (D). CVs of 50 μ M catechol at SWNTs/GCE in the absence (curve a) and the presence of 100 μ M GSH (curve b). Buffer, 0.1 M PBS (pH 7.4). Scan rate, 50 mV s^{-1}

Scheme 1 Proposed reaction pathway (A) for the addition of Cys to the catechol-analogue derivative via the electro-catalytic mechanism (B) for the addition of GSH to the catechol-analogue derivative via the polymerization-adduct ECE mechanism at SWNTs /GCE.

Fig. 2 TEM images of (A, B) SWNTs and (C, D, E) SWNTs after electrochemical treatment with the catechol, respectively.

A. Catechol-Analogue Indicators

Fig. 3 (A) Structure of redox mediators. (B) CVs obtained at SWNTs /GCE in 0.1 M PBS (pH 7.4) containing of 100 μM 4-nitrocatechol and different concentration of GSH (curve a to d: 0, 100, 300, 600 μM). (C) CVs obtained at SWNTs /GCE in 0.1 M PBS (pH 7.4) containing of 100 μM dopamine and different concentration of GSH (curve a to e: 0, 100, 300, 600, 800 μM). (D).CVs obtained at SWNTs /GCE in 0.1 M PBS (pH 7.4) containing 50 μM catechol and different concentration of GSH (0 μM , black; 25 μM , red; 50 μM , blue; 75 μM , green; 100 μM , magenta; 125 μM , yellow; 150 μM , cyan; 250 μM , navy; 350 μM , gray; 400 μM , pink). Scan rate, 50 mV s⁻¹

Fig. 4 (A) CVs obtained in different pH buffer solution containing 50 μM catechol at SWNTs /GCE. From left to right: 0.1 M $\text{NH}_4\text{Cl-NH}_3$ buffer (pH 12.0, curve a; pH 10.0, curve b), 0.1 M PBS (pH 7.4, curve c), 0.1 M HAc-NaAc buffer (pH 5.0, curve d), 0.1 M H_2SO_4 solution (pH 1.0, curve e). (B) CVs obtained in different pH buffer solution containing 50 μM catechol and 100 μM GSH at SWNTs/GCE. curve a: 0.1 M PBS (pH 7.4), curve b: 0.1M HAc-NaAc buffer (pH 5.0), curve c: 0.1 M H_2SO_4 solution (pH 1.0). Scan rate, 50mV s^{-1}

Fig. 5 CVs obtained at SWNTs /GCE in 0.1M PBS (pH 7.4) containing 50 μM catechol and different compounds: (A) Cys (curve a to d: 0, 50, 150, 300 μM). (B) Hcy (curve a to j: 0, 5, 12, 25, 50, 75, 100, 150, 200, 250 μM). (C) MCH (curve a to f: 0, 50, 100, 150, 250 μM). (D) DTT (curve a to e: 0, 50, 100, 150, 250 μM). (E) MUA (curve a to f: 0, 50, 150, 300, 400, 500 μM). (F) Cym (curve a to e: 0, 50, 150, 300, 400 μM). Scan rate, 50 mV s^{-1}

Fig. 6 CVs and plot of I_p (μA) against C_{GSH} at SWNTs /GCE in different concentrations of GSH and catechol. (A) Low concentration of GSH (curve a to h: 0, 5, 10, 25, 50, 70, 90, 100 μM) in the presence of 50 μM catechol. (B) High concentration of GSH (curve a to j: 100, 150, 200, 250, 300, 350, 400, 450, 500, 0 μM) in the presence of 50 μM catechol. (C) The concentration of GSH (curve a to j: 0, 7,

15, 30, 50, 75, 85, 100, 125, 150 μM) in the presence of 75 μM catechol. (D) The concentration of GSH (curve a to h: 0, 1, 2, 3, 6, 12, 14, 20 μM) in the presence of 10 μM catechol. Buffer, 0.1M PBS (pH 7.4). Scan rate, 50mV s⁻¹

Fig. 7 (A) Typical CVs obtained in 0.1M PBS (pH 7.4) including 10 mM glucose which did not incubate (curve b) and incubate Hela cells (1×10^6 cells•mL⁻¹) without (curve c) and with (curve d) 15 μg /mL doxorubicin treatment after 1 h in the absence (curve a) and presence of 50 μM catechol (curve b, c, d) at SWNTs /GCE. Scan rate, 50 mV s⁻¹. Bright field pictures for HeLa cells on the glass substrate without (B) and with (C) 15 μg /mL doxorubicin treatment after 1 h.

Table 1 Analytical performance for the analysis of GSH

Matrix	Method	Electrode material	E _{det}	LOD	Sample analysis	Ref.
0.1 M PBS(pH 7.4) 70:30 MeOH / PBS(0.05M, pH 7.3)	AMP	The Pt ₂₃ -NiCo /GCE	0.0V vs.Ag /AgCl	20 nM	liver cancer cells	20
	CV	GCE using catechol mediator	+0.42 V vs. Ag/AgCl	-	N/A	19
0.1 M PBS (pH 7.0)	SWV	FePt/CNTs/CPE using catechol mediator	+0.41 V vs. Ag/AgCl	1 nM	Hemolyzed erythrocyte, Tablet, Urine	25
0.1M PBS (pH 7.0)	SWV	CPE modified with benzamide derivative and MWNTs	+0.33 V vs. Ag/AgCl	0.05μM	Hemolyzed erythrocyte, Tablet, Urine	27
Tissue culture medium	CV	GCE using catechol mediator	+0.2 V vs.SCE	1μM	N/A	40
0.1M phthalate buffer (pH 3.45)	AMP	GCE modified with PPy incorporated with PQQ	+0.50 V vs. Ag/AgCl	13.2μM	N/A	41
0.02M PBS (pH 6.5)	DPV	NiHCF/CTAB/Au NPs Pt UME	+0.58 V and +0.45 V vs.Ag/AgCl	0.08μM	N/A	28
0.1M acetate buffer (pH 5.0)	AMP	Reduced GO/GCE	+0.23 V vs. Ag/AgCl	5 μM	eye drops	21
0.1M PBS (pH 7.0)	SWV	BPPG electrode with nitrophenyl groups	-0.1 V vs.SCE	8.1 μM	N/A	42
0.1M PBS (pH 7.0)	SWV	ZnO/ MWNTs / CPE nanocomposite using BCB mediator	+0.4 V vs. Ag/AgCl	0.002 μM	Hemolyzed erythrocyte, Tablet, Urine	30
0.1M PBS (pH 5.0)	AMP	MWNTs modified GCE	+0.30 V vs. Ag/AgCl	3.3 μM	N/S	43
0.1M PBS (pH 5.0)	CHR	MWNTs paste electrode with 3,4-dihydroxy-cinnamic acid as mediator	+0.26 V vs. Ag/AgCl	0.1 μM	Hemolyzed erythrocyte, Tablet, Urine	44
0.1M PBS (pH 7.0)	CV	SWNTs / GCE using catechol mediator	-0.16V vs. Ag/AgCl	0.5 μM	Hela cells	This work

Where E_{det} = Detection Potential, LOD= Limit of Detection, PBS= Phosphate-buffered saline, vs.= versus, N/A = Not Applicable, SCE= Saturated Calomel Electrode, CV =Cyclic Voltammetry, DPV =Differential Pulse Voltammetry, CHR = Chronoamperometry, SWV = Square Wave Voltammetry, AMP = Amperometry, GCE = Glassy Carbon Electrode, CP = carbon paste electrode, BPPG =

Basal plane pyrolytic graphite electrode, BCB = 4,5-bis(4-chloroanilino)-1,2-benzendiol, SWNTs = Single-walled carbon nanotube, MWNTs = Multiwall carbon nanotube, PPy = polypyrrole, PQQ = pyrroloquinoline quinone, GO = Graphene, UME = ultramicroelectrode, NiHCF = nickel hexacyanoferrate, AuNPs = Au nanoparticles, CTAB = cetyltrimethylammonium bromide.