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Short communication

Electrochemical Detection of Low-Molecular-Mass Thiols Based on the Cleavage of Disulfide Bond on Gold Electrode

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In this work, we reported a simple and sensitive electrochemical method for the detection of low-molecular-mass thiols based on the disulfide cleavage on electrode. Specifically, oxidized form of glutathione (GSSG) was immobilized onto the gold electrode, followed by the derivation with electrochemically active ferrocenecarboxylic acid. A couple of redox wave from the oxidation/reduction of ferrocene (Fc) moieties was observed. The cleavage of GSSG through the thiol-disulfide exchange reaction induced the loss of Fc groups and the decrease in the current. As a result, a relatively low detection limit of 20 pM was achieved. Moreover, protein thiols (e.g. bovine serum albumin or BSA and metallothioneins or MTs) and other small biological molecules (e.g. dopamine, glucose, ascorbic acid and uric acid) have no impact on the detection assay.

Keywords: low-molecular-mass thiols; electrochemistry; disulfide cleavage; self-assembled monolayers; gold electrode

1. INTRODUCTION

Low-molecular-mass thiols including homocysteine (Hcy), cysteine (Cys), cysteinylglycine (CysGly) and glutathione (GSH) are cellular components that play critical functions in metabolism and homeostasis [1,2]. They are important in regulating the biological activity of protein and in the cellular antioxidant defense system [3]. Also, they are widely used in the food industry as antioxidants and in the pharmaceutical industry as biomarkers [4]. To date, considerable attention has been drawn to the detection of thiols in biological, medical, and clinical studies with UV-Vis spectrophotometry, high performance liquid chromatography (HPLC), fluorescence and electrochemistry [2,4-8]. Among them, spectrophotometric thiol assay employing electrophilic disulphides, such as Ellman's reagent (5,5′-

dithio-bis(2-nitrobenzoic acid), DTNB) or 4,4′-dithiodipyridine (DTDP), are commonly utilized to measure both protein sulphydryls and low-molecular-mass thiols [9,10]. This method is rapid and simple, but is less sensitive and lacking of selectivity for detection of low-molecular-mass thiols. Alternately, electrochemical strategies have been reported for thiols detection in view of their advantages of simplicity, rapidity, high sensitivity and capacity of being readily integrated with other techniques for multi-analysis. However, the determination of thiols at the electrodes (e. g. carbon, glassy carbon, gold, platinum, graphite, and silver) has experienced great challenges for assay of real sample because of the slow electron-transfer of the electrochemical reaction, the high overpotentials required and the strong thiols adsorption on different electrode materials [4,11-14]. These drawbacks also make the study of their redox behavior and selectivity towards interferents such as ascorbic acid (AA) and uric acid (UA) being unsatisfactory. Thus, there remains significant room for the development of simple, sensitive and selective electrochemical approaches for detection of low-molecular-mass thiols.

Among kinds of electrochemical methods, redox tags-labeled peptide/oligonucleotide probes site-specifically attached to an interrogating electrode is one of the most attractive approaches. Change in the configuration of peptide/oligonucleotide or decrease in the amount of redox tags leads to a readily detectable change in Faradaic current upon voltammetric interrogation. Based on this principle, many electrochemical sensors have been developed for analysis of proteases, DNA, metal ions and small molecules [15,16]. It is well known that the disulfide bridge in a steric environment is accessible to reduction by a small thiol-containing molecule through the thiol-disulfide exchange reaction [17,18]. For this reason, we suggested that low-molecular-mass thiols could be determined on the electrode covered with redox tags-labeled disulfide-containing probe based on thiols-induced cleavage of disulfide bond. Such cleavage would result in the remove of redox tags and the decrease of the Faradaic current. In this work, we demonstrated the feasibility by employing ferrocene (Fc)-labeled disulfide-modified gold electrode for detection of thiols. Further, selectivity and sensitivity of the method were demonstrated by testing different sulphydryl-containing species and small biological molecules.

2. EXPERIMENTAL

2.1 Chemicals and reagents

Reduced and oxidized forms of glutathione (GSH and GSSG, respectively), 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC), N-hydroxybenzotrizole (HOBT), bovine serum albumin (BSA), N-hydroxysulfosuccinimide (NHSS), dicyclohexylcarbodiimide (DCC), dithiothreitol (DTT), cysteamine hydrochloride, DTDP, 3-mercaptopropionic acid (MPA), K_2HPO_4, KH_2PO_4 and L-cysteine were obtained from Sigma–Aldrich. Homocysteine and N,N-dimethylformamide (DMF) was purchased from Sangon Biotech. Co., Ltd. (Shanghai, China). Metallothioneins (MTs) was acquired from Hunan Lugu Biotech Co., Ltd (Changsha, China). Ferrocenecarboxylic acid (FcA) were provided by XiYa Chemical Reagent Co. (Chengdu, China). All

other reagents were analytical-grade reagents and used without further purification. The thiols stock solutions were prepared freshly with N_2-saturated deionized water and diluted to the desired concentration with N_2-saturated phosphate buffer solution (PBS, 10 mM, pH 7.4) before use.

2.2 Procedures

Prior to each measurement, the gold disk electrodes were polished with diamond pastes down to 3 μm and alumina pastes down to 0.3 μm and subsequently sonicated in water. The MPA self-assembled monolayers (SAMs) were formed by immersing the cleaned electrodes in ethanol solutions containing 10 mM MPA for 12 h. Then, the electrodes were rinsed with ethanol/water to rid any non-specifically adsorbed substance. GSSG was immobilized onto gold electrodes by cross-linking GSSG molecules onto the MPA SAMs surface via the EDC/NHSS-mediated amine coupling reaction [19]. Briefly, MPA-covered electrodes were soaked in a solution comprised of 0.2 M EDC and 0.1 M NHSS for 30 min, followed by incubating the electrodes with 0.1 mM GSSG (pH 7.4) for 5 h to form the GSSG-covered surface. For the coupling of ferrocenecarboxylic acid, ferrocenecarboxylic acid at the concentration of 1 mM was first activated by 1 mM DCC/HOBT for 30 min; then, the GSSG-covered electrodes were incubated with the mixed solution for extra 30 min and rinsed with DMF and deionized water. For the assay of thiols, the modified electrodes were incubated with different concentrations of thiols for 5 min; then, electrochemical determination was performed on a CHI 660E electrochemical workstation (CH Instruments, Shanghai, China) in a homemade plastic three-electrode cell with a platinum wire as the auxiliary electrode and an Ag/AgCl as the reference electrode. The supporting electrolyte was 10 mM PBS containing 50 mM Na_2SO_4.

To evaluate the accuracy of the electrochemical assay, the content of thiols was measured by the standard spectrophotometric assay with a Cary 50 spectrophotometer. Briefly, thiols were mixed with pH 7.0 PBS containing 0.1 mM DTDP for 5 min. Then, 2 mL of the mixed solution was transferred to a 1 cm quartz spectrophotometer cell. The absorbance of the 4-thiopyridone (4-TP) product at 324 nm was measured and the concentration of thiols was calculated with the extinction coefficient (ε) of 21400 M^{-1} cm^{-1} at 324 nm [10].

3. RESULTS AND DISCUSSION

3.1 Principle and feasibility of the method

The principle of our strategy is shown in Fig. 1A. Glutathione oxidized (GSSG) was immobilized onto the gold electrode and then derivatized with ferrocenecarboxylic acid through the amine coupling reaction (Sample 1). A couple of redox wave from the oxidation/reduction of ferrocene (Fc) moieties can be observed. The cleavage of GSSG by thiols through the thiol-disulfide exchange reaction will induce the loss of Fc groups and the decrease in the redox current (Sample 2). To demonstrate the feasibility of the method for the detection of thiols, GSH was first tested. The CVs showed in Fig. 1B were collected at the MPA/GSSG/Fc-covered gold electrode in PBS solution before

and after treatment with GSH. The redox peaks with Epa = 0.385 V and Epc = 0.305 V were attributed to the oxidation/reduction of Fc (black curve). The current dropped almost to the background level after the electrode was incubated with GSH (red curve), indicating that the disulfide was cleaved by GSH.

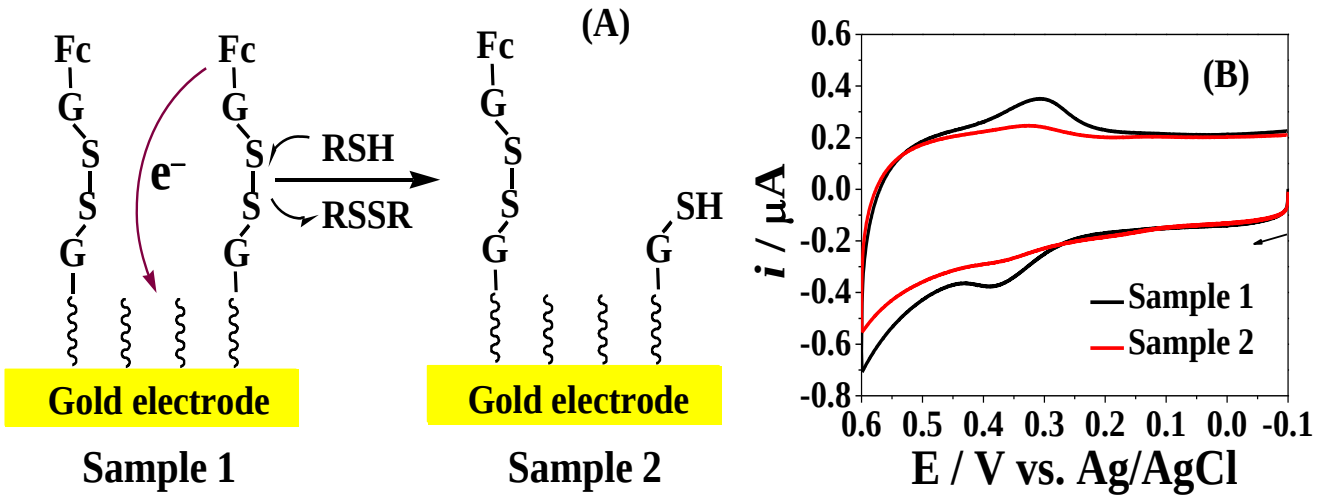

Figure 1. (A) Scheme representation showing the strategy for thiols detection and (B) CVs collected at the MPA/GSSG/Fc-covered gold electrode before (black) and after (red) treatment with 5 nM GSH.

3.2 Dependence on GSH concentration

Differential pulse voltammetry can decrease the background charging currents and in turn increase the detection sensitivity [20]. To demonstrate the sensitivity of this method, differential pulse voltammograms (DPVs) at the MPA/GSSG/Fc-covered gold electrode were collected after treatment with different concentrations of GSH. As shown in Fig. 2A, the oxidation peak current decreased with the increase of GSH concentration, demonstrating that the cleavage of GSSG depends upon GSH concentration. Thus, the level of GSH could be determined by the current-concentration curve. The anodic current change Δi_{pa} (i_0–i, where i_0 and i represent the anodic current in the absence and presence of GSH, respectively) was used here to evaluate the analytical merits. The dependence of the anodic current on the GSH concentration is presented in Fig. 2B. Δi increased linearly with the concentrations of GSH ranging from 0.05 nM to 2.00 nM (see the inset in Fig. 2B). The linear regression equation is expressed as Δi_{pa} (μA) = 0.019 + 0.096 [GSH] (nM) (R^2 = 0.98). The detection limit (3s) of the method was determined to be 20 pM, which is lower than those achieved by fluorescence, UV-Vis spectroscopy and other electrochemical methods [4,6,8,9]. Moreover, we believe that the sensitivity would be further improved by signal amplification of nanoparticles in such detection format.

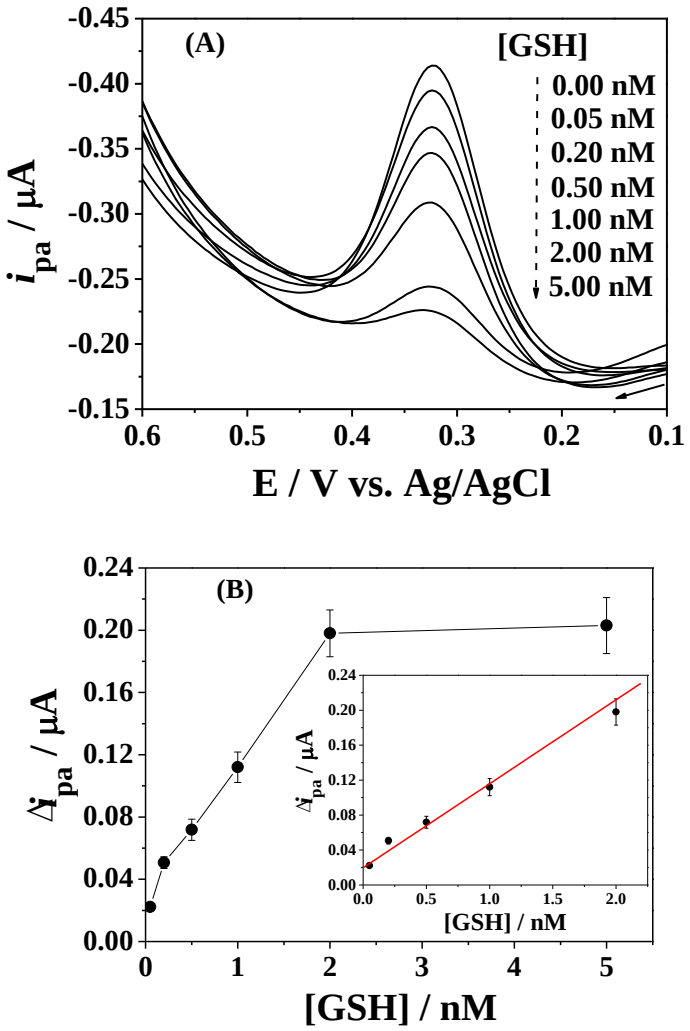

Figure 2. (A) DPVs collected at the MPA/GSSG/Fc-covered gold electrode after treatment with different concentrations of GSH. (B) Dependence of the anodic current change (Δi_{pa}) on the final concentration of GSH. The absolute errors were deduced from at least three replicate measurements and are shown as the error bars.

3.3 Selectivity to GSH

The selectivity of the assay was evaluated by challenging it with other thiol compunds varying in molecular weight and non-thiol compounds, such as ascorbic acid (AA), dopamine (DA), glucose, and uric acid (UA). As shown in Fig. 3, the non-thiol compounds did not cause significant change in the anodic current. This result is acceptable because these compounds are unable to break up the disulfide bond, although some of them have high reducibility. Interestingly, we found that the sensor is selective to thiols with low molecular weight (e. g. cysteamine, cysteine, DTT, GSH and Hcy), and is less sensitive to protein thiols (BSA and MTs). The result indicated that the disulfide can not be cleaved by protein thiols, which is in good agreement with that reported previously [21]. We presume that this behavior is caused by their poor reactivity to the immobilized GSSG due to the ortho-effect of protein. Moreover, low-molecular-mass thiols are susceptible to oxidation by oxygen. Here, the stability of these tested low-molecular-mass thiols in the oxygen atmosphere is also examined. As a

result, we found that the thiols caused a less anodic current change in O_2-saturated solution in comparison with that in N_2-saturated solution, indicating that part of thiols were oxidized by O_2. To compare the method with well-established techniques, we also investigated the effect of O_2 on the stability of thiols by the standard spectrophotometric assay. The amount of thiols was found to decrease after six-hours' incubation with O_2, which is in agreement with the electrochemical assay data.

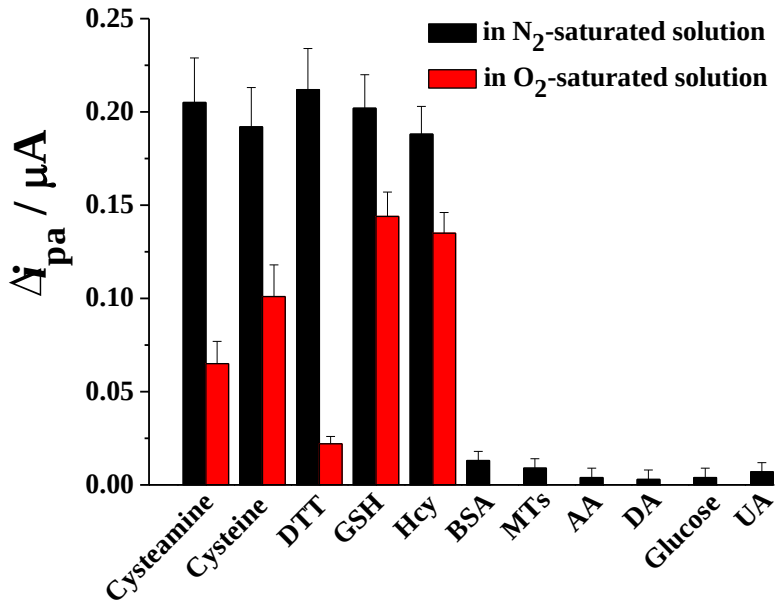

Figure 3. Selectivity of the sensing system. The final concentrations of the tested compounds are 2 nM.

4. CONCLUSION

In summary, we reported a simple and sensitive electrochemical strategy for the detection of thiols based on the disulfide cleavage on electrode. The results indicated that the disulfide bond was accessible to reduction by low-molecular-mass thiols but not protein thiols. Moreover, other small biological molecules, such as dopamine, glucose, ascorbic acid and uric acid, have no interference in the detection assay. The detection limit of 20 pM is lower that those achieved by fluorescence, UV-Vis spectroscopy and other electrochemical methods. We anticipate that the proposed electrochemical sensor would become a protocol for the detection of low-molecular-mass thiols in clinical diagnostics and laboratory research.

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