Int. J. Electrochem. Sci., 8 **(2013)** 8227 - 8235

International Journal of
ELECTROCHEMICAL
SCIENCE
www.electrochemsci.org

Short Communication

Study on the Interaction of Metallothionein with Glutathione Using Ferrocenyl-labeled Glutathione as an Electrochemical Probe

Ping Chen[1,2], *Yong Peng*[1], *Yuan-Qiang Hao*[1], *You-Nian Liu*[1,3,*], *Juan Li*[1,3]

[1] College of Chemistry and Chemical Engineering, Central South University, Changsha, Hunan 410083, P. R. China
[2] College of Chemical and Biological Engineering, Changsha University of Science and Technology, Changsha, Hunan 410114, P.R. China.
[3] Key Laboratory of Resource Chemistry of Nonferrous Metals(Central South University), Ministry of Education, Changsha, Hunan 410083, P. R. China.
[*]E-mail: liuyounian@csu.edu.cn

Received: 9 April 2013 / *Accepted:* 14 May 2013 / *Published:* 1 June 2013

The interaction between glutathione (GSH) and metallothionein (MT) plays a critical role in metal release from MT in life processes. In this paper, the interaction was measured directly by voltammetric method at the MT-modified electrode, due to the attachment of the redox-active ferrocene moiety to GSH. Particularly, the covalent attachment of ferrocene moiety to GSH does not significantly perturb the GSH structure and its binding behavior with MT. The GSH/MT stoichiometry and binding constant *K*a were obtained by cyclic voltammetric method and the values are 1.78 ± 0.15 and 5.44 ± 0.43 $\times 10^6$ M^{-1}, respectively. In addition, zinc release from MT in the presence of glutathione or glutathione disulfide (GSSG) was also studied by means of differential pulse voltammetry. The difference in binding constant between Zn-MT and GSH-MT complexes explains the inhibition of GSH on the zinc release from MT.

Keywords: Ferrocenyl-labeled glutathione; metallothionein; interaction behavior; zinc ion release

1. INTRODUCTION

Metallothionein (MT), a low molecular-weight and cysteine-rich protein, is composed of an *N*-terminal, nine cysteine residues and three metal clusters and a *C*-terminal, eleven cysteine residues and four-metal cluster[1-4]. It is considered that each metal binds with four cysteine residues to form metal clusters[5]. Zinc, one of the metals normally bound to MT, is an important constituent of many

proteins and enzymes[6, 7]. Zn_7-MT is the main source of zinc in the human body and zinc ions can be released from Zn_7-MT by the small oxidizing molecules such as hypochlorous acid, peroxide and superoxide, or at low pH [8, 9]. Zinc ions in the Zn_7-MT can also be exchanged with other metal ions such as Hg(II), Cd(II), and Cu(I) [5, 10-12]. These metal ions play important roles as essential or toxic elements in life processes [13, 14].

Glutathione (GSH), an intracellular tripeptide, is composed of glutamine, cysteine and glycine, and is present at high abundance in plants, mammals, fungi and prokaryotic organisms. It plays important roles in biological reactions as diverse as antioxidation, detoxification of heavy metals and pernicious electrophilic species, and regulations of gene and protein expressions[15]. It is well-known that glutathione disulfide (GSSG) and GSH are the critical modulators in the zinc ions transfer and release process from MT. For example, Brouwer et al. found GSH, in concern with MT, regulates essential metals and detoxify heavy metals[16]. They demonstrated that CdZn-MT can bind with GSH, locating on MT's zinc-containing *N*-terminal domain according to the energy minimized by molecular mechanics calculations[16].Vallee and coworkers reported that GSH can inhibit zinc transfer from MT molecule, but facilitate the interaction between MT and GSSG [9]. Later, they demonstrated that GSSG can facilitate the zinc transfer rate for human MT [17]. However, the binding of MT with GSH is still unclear and no direct methods are reported to monitor the interaction between MT and GSH and the zinc release process from MT in the presence of GSH or GSSG.

Up to now, there are only very few papers focused on the interaction of MT with GSH, due to the lack of effective biomarkers. Generally, Ellman's reagent (5,5'-dithiobis(2-nitrobenzoic acid)) was utilized to label the sulfhydryl involved in the reaction[18]. However, the introduction of a foreign compound via the sulfhydryl groups may affect the biological reactions involving GSH or prevent the binding of GSH to a biological species from being accurately and rapidly measured. In addition, Jiang et al. introduced radiolabeled method to determine the zinc transfer from MT to zinc-depleted orbital dehydrogenase modulated by GSH/GSSG [9], but this method is labor-intensive due to requiring for additional separation and has the safety problems associated with radioactive material.

In the past years, electrochemical methods have been employed as a viable means to study certain properties of MT because the complexes of cysteine residues of MT and some metal ions, such as Cd, Hg, Ag and Cu ions are electroactive[19-22]. On the other hand, direct detection of the interactions in biological process by using an electrochemical probe is reported [23], which can provide a good sensitivity and simplicity of use. The probe can be readily formed by attachment of electroactive compounds to the biologically important substances. Recently, we synthesized glutathione-ferrocene(GSH-Fc), and utilized it as an electroactive marker to probe the interaction between GSH and bovine serum albumin[24].Our work confirmed that labeling GSH molecule with a ferrocene moiety at the *C*-terminal retains the biological activity of GSH, and found that the attached ferrocene moiety does not alter the binding properties between GSH and bovine serum albumin.

In this work, we are trying to employ GSH-Fc as an electrochemical probe to investigate the interaction between MT and GSH, and to gain an insight into the impact of GSH on the zinc release from MT by electrochemical methods.

2. EXPERIMENTAL

2.1 Reagents and chemicals

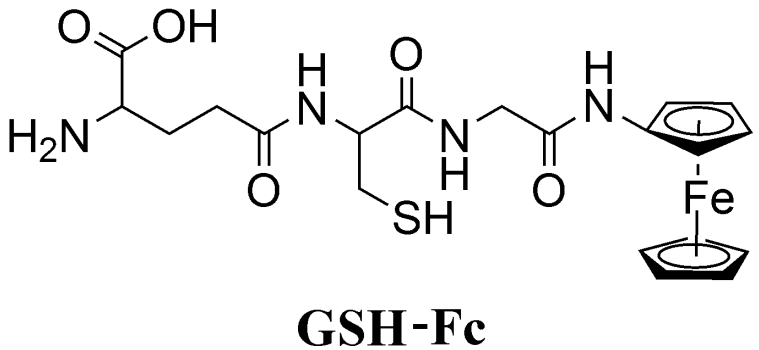
Glutathione (GSH), glutathione disulfide (GSSG), 11-mercaptoundecanoic acid (MUA), *N*-hydroxysuccinimide (NHS), and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were purchased from Thermo-Fisher Scientific (Pittsburgh, PA, USA). The rabbit liver MT-II was supplied by Lugu Biotech Co. (Changsha, China). Aqueous solutions were prepared using Millipore water (18 MΩ·cm, Simplicity Model, Billerica, MA, USA).

2.2 Apparatus

Electrochemical experiments were carried out using a CHI-660 electrochemical workstation (CH Instruments, Austin, TX, USA). A three-electrode system was used in the measurements, comprising a 2 mm diameter bare glass carbon or MT-modified 2 mm diameter gold electrode as the working electrode, a platinum flag auxiliary electrode and an Ag/AgCl reference electrode. The buffer solution was 0.1 M Tris-HCl (pH 7.4), the supporting electrolyte was 0.1 M NaCl.

2.3 Preparation of glutathione-ferrocene conjugate and the MT-modified electrode

Glutathione-ferrocene (GSH-Fc) was prepared following our previous procedure (Scheme 1)[24].To obtain a stable MT-modified gold electrode, MUA self-assembled monolayers (SAMs) were firstly immobilized on the surface of the gold electrode. The bare gold electrode was polished with 0.5 um a-Al_2O_3 slurry on microcloth pads (Buehler, Lake Bluff, IL, USA) and rinsed thoroughly with water. The electrode was then immersed in 10 mM MUA dissolved in ethanol for 18 h. The resultant MUA-modified electrode was washed successively by ethanol and water and dried under nitrogen. The electrode was incubated with 10 uL Tris-HCl buffer solution containing EDC/NHS (5 mM each) for 1 h. Upon rinsing and drying, the activated MUA film was allowed to react with 10 uL of 5 mM MT solution for 4 h.



GSH-Fc

Scheme 1. Structure of ferrocenyl-labeled glutathione.

3. RESULTS AND DISCUSSION

3.1 Electrochemical behaviors of GSH-Fc at the MT-modified electrode

Cyclic voltammetry (CV) was employed to determine the electrochemical properties of GSH-Fc in a Tris-HCl buffer solution (pH7.4) at the bare glass carbon electrode firstly. As can be seen from curve a in Fig.1, a pair of well-defined voltammetric peaks, with the cathodic (E_{pc}) and anodic peak potentials (E_{pa}) at 0.205 and 0.147 V (vs. Ag/AgCl), respectively, were observed for GSH-Fc on the naked gold electrode. The ratio of oxidative to reductive peak currents is 1.21 and the peak currents were found to be proportional to the square root of scan rate (data not shown), suggesting that GSH-Fc undergoes a virtually reversible electron transfer reaction in solution [25]. For comparison, the electrochemical properties of 0.1 mM GSH-Fc in a Tris-HCl buffer solution (pH7.4) at an MT-modified gold electrode was also investigated (*cf*. dashed curve in Fig.1b). Likewise, a couple of well-defined peaks, with the anodic peak potential at 0.218 V and the cathodic peak potential at 0.154 V (vs. Ag/AgCl), were observed.

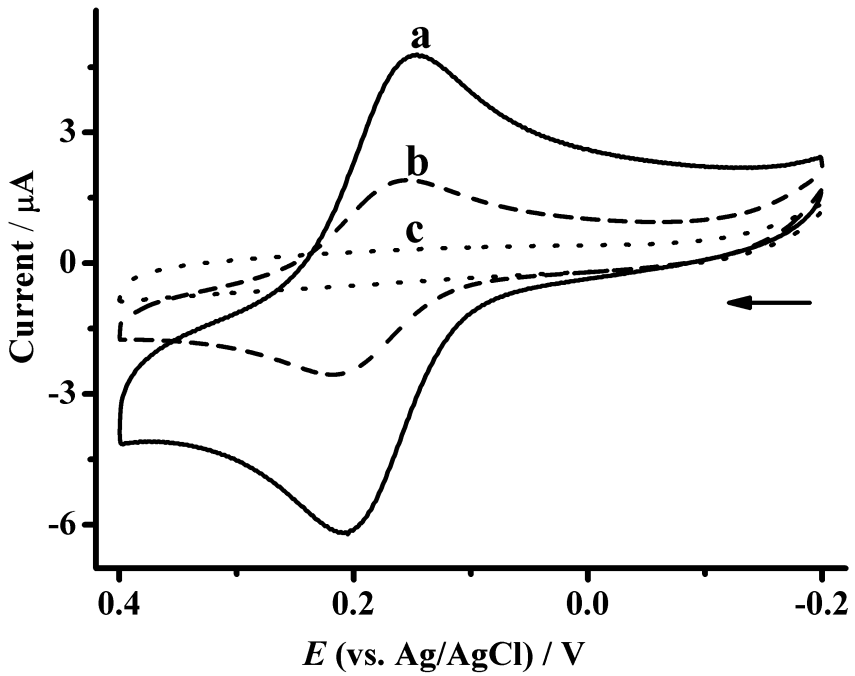


Figure 1. Cyclic voltammograms of 0.1 mM GSH-Fc in 0.1 MTris-HCl buffer solution(pH 7.4) at a bare glass carbon electrode (a, solid curve) and at an MT-modified electrode (b, dashed curve); and cyclic voltammogram of 0.1 M $[Fe(CN)_6]^{3-}$solution at an MT-modified electrode (c, dotted curve). The scan rate was 100 mV/s and the arrow indicates the initial scan direction.

In order to obtain more details of the MT-modified electrode, the redox probe $[Fe(CN)_6]^{3-}$ was employed to explore the properties of the MT films on the electrode. As shown in Fig.1c, the cyclic voltammogram of 1.0 mM $[Fe(CN)_6]^{3-}$ in 0.1 M KCl solution at the MT-modified electrode exhibits no redox peaks at the redox potential from –0.2 V to 0.4 V of the probe molecule, implying that no

$[Fe(CN)_6]^{3-}$ could reach the surface of the MT-modified gold electrode, *i.e.*, the film is electrically denser. This can be contributed by the covalent linkage between MT and the gold electrode. In addition, we performed another cyclic voltammetry at MUA-modified gold electrode in 0.1 mM GSH-Fc solution. As expected, no redox peak appeared in the range from –0.2 V to 0.8 V (data not shown), demonstrating that there exists no electron transfer between GSH-Fc and MUA layers on the electrode. Therefore, it is concluded that specific interactions between GSH-Fc and MT are responsible for the observed behavior.

3.2 Determination of the stoichiometry and binding constant of MT with GSH-Fc

As shown in Fig. 2, the peak currents decreased with the addition of MT. Such a decrease in current can be attributed to the formation of a complex of MT and GSH-Fc. The results are consistent with those obtained previously on interaction between the electroactive small molecules with proteins[26-28]. Although the complex of GSH-Fc and MT were formed, the electrochemical parameters do not change significantly (*cf.* Table 1). Therefore, the MT/GSH-Fc binding sites and constant can be determined by recording the voltammogram of different concentrations of GSH-Fc in the presence of MT. Assuming that one MT molecule binds to *m* GSH-Fc molecules, the equilibrium equation can be expressed as follows[24]:

m GSH-Fc + MT $\rightleftharpoons$ $MT(GSH\text{-}Fc)_m$

The corresponding equilibrium constant K_a is

$$K_a = [MT(GSH\text{-}Fc)_m] / [GSH\text{-}Fc]^m [MT] \quad (1)$$

and the mass balance equation is

$$[MT]_0 = [MT] + [MT(GSH\text{-}Fc)_m] \quad (2)$$

where $[MT]_0$, $[MT]$ and $[MT(GSH\text{-}Fc)_m]$ are corresponding to the total, free and bound concentration of metallothionein in the solution, respectively. Thus, the peak current difference (ΔI_p) of GSH-Fc in the absence and presence of MT should be proportional to the concentrations of the complex of $MT(GSH\text{-}Fc)_m$,

$$\Delta I_p = k\,[MT(GSH\text{-}Fc)_m] \quad (3)$$

At the stoichiometric GSH-Fc/MT binding concentration, the maximum peak current difference $\Delta I_{p,max}$ is also related to the total concentration of MT,

$$\Delta I_{p,max} = k[MT(GSH\text{-}Fc)_m]_{max} = k[MT]_0 \quad (4)$$

Combining equations (1) to (4), the following equation can be derived:

$$\log [\Delta I_p/(\Delta I_{p,\,max} - \Delta I_p)] = \log K_a + m \log [GSH\text{-}Fc] \quad (5)$$

Curve a in Fig.3 is the plot of the anodic peak current of free GSH-Fc against the concentration of GSH-Fc, and curve b corresponds to the currents when 20 uM MT was present. Notice that free GSH-Fc current increases linearly with the GSH-Fc concentration, whereas two lines of different slopes were observed when MT was present in the solution. Indeed, the plot of $\log[\Delta I_p/(\Delta I_{p,\,max}-\Delta I_p)]$ vs. log[GSH-Fc] is linear with the regression equation of $\log[\Delta I_p/(\Delta I_{p,\,max}-\Delta I_p)]$ (uA) = 6.74+ 1.78 log[GSH-Fc](M) (R^2=0.992) (see the inset in Fig.3). The binding constant (K_a) and the GSH/MT stoichiometry (m) could be calculated and the values are $5.54 \pm 0.43\times10^6$ M^{-1} and 1.78 ± 0.15,

respectively. One the other hand, the binding constant of Zn-MT complex is 3.2×10^{13} M^{-1} at pH 7.4 [11], much higher than that of GSH-MT complex. The big difference in binding constant between Zn-MT and GSH-MT complexes explains that why zinc can hardly be released from MT in the presence of GSH(GSH-Fc).

Table 1. Cyclic voltammogram of 20 uM GSH-Fc solution at the bare glass carbon electrode in the absence and presence of 20 uM MT.

	E_{pa}/V	E_{pc}/V	□E/V	I_{pa}/I_{pc}
GSH-Fc	0.217	0.154	0.063	1.03
GSH-Fc+MT	0.217	0.152	0.065	1.02

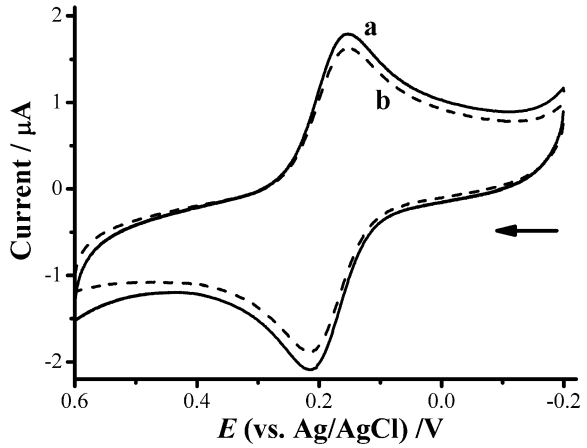


Figure 2. Cyclic voltammograms of 20 uM GSH-Fc solution in the absence (a, solid curve) or presence (b, dashed curve) of 20 uM MT. The scan rate was 100 mV/s and the arrow indicates the initial scan direction.

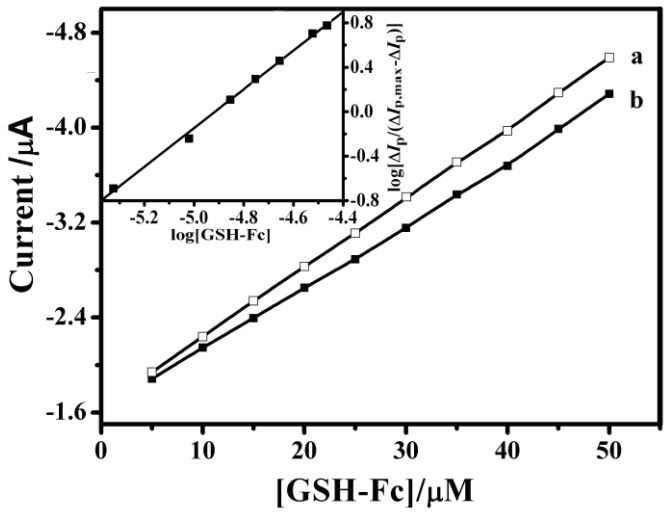


Figure 3. Plots of the GSH-Fc anodic peak currents vs. [GSH-Fc] in the absence (a)and presence (b) of 20 mM MT solution. Inset: The plot of $\log[\Delta I_p/(\Delta I_{p,max}-\Delta I_p)]$ vs.log[GSH-Fc].

3.3 Investigation of the behavior of zinc release from MT in the presence of GSH or GSSG

It is well-known that there are seven zinc ions in one MT molecule, and all zinc ions bind with residual thiols of cysteine[29]. The zinc release from MT is prohibited in the presence of GSH, but is promoted in the presence of GSSG. Here, the differential pulse voltammetry (DPV) was employed to investigate the zinc ion release from Zn-MT molecules in the presence of GSH or GSSG. As shown in Fig. 4 (solid curve), there was a remarkable peak of MT-modified electrode at the potential of -1.03 V (vs. Ag/AgCl), which is owing to the zinc ion reduction in the MT molecules. In the presence of a 50 uM GSH-Fc solution, the zinc reduced peak current does not change at the MT-modified gold electrode, indicating that GSH inhibits the zinc release from Zn-MT. However, the reduced peak current of zinc ion decreased in 0.1 mM GSH-Fc solution with the addition of GSSG (*cf.* dotted curve in Fig. 4), suggesting that the presence of GSSG can facilitate zinc release from Zn-MT.

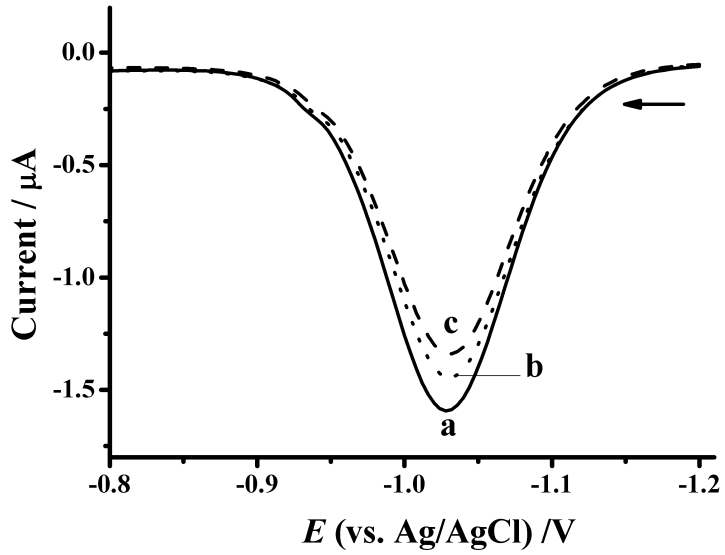


Figure 4. Differential pulse voltammogram (DPV) of 50 uM GSH-Fc at the MT-modified electrode in 0.1 M Tris-HCl buffer solution (a, solid curve), in the presence 50 uM (b, dotted curve) and 100 uM (c, dashed curve) GSSG. The arrow indicates the initial scan direction.

We calculated the maximum charge difference of MT-modified electrode in the presence of GSSG, and found the value is one seventh related to the charge of original MT-modified electrode[30]. The results revealed that one zinc ion per MT molecule is released. Furthermore, when the MT-modified electrode was immersed to GSH/GSSG mixture solutions, the results were similar to those of GSH-Fc/GSSG mixture solutions (data not shown). It also suggests that the attaching of the ferrocene moieties does not affect the GSH binding with MT. It should be pointed that the peak currents of ferrocene moiety at potential of 0.2 V(vs. Ag/AgCl) do not alter when addition of GSSG to GSH-Fc solution, demonstrating that the complex of MT with GSH-Fc (or GSH) are not dissociated when zinc is released from Zn-MT.

4. CONCLUSIONS

Interaction between MT and GSH is vital in biological processes. In this work, we utilized ferrocenyl-labeled GSH as an electrochemical probe to monitor the interactions between GSH and MT by voltammetric methods in solution or on the MT modified gold electrode, respectively. The number of binding sites and binding constant was determined by voltammetric method. In addition, the ferrocenyl-labeled GSH was employed to investigate the zinc release from Zn-MT molecule in the presence of GSH or GSSG by differential pulse voltammetry. Our work confirmed that labeling GSH molecule with a ferrocene moiety at the *C*-terminal retains the biological activity of GSH, and can be used to investigate the interaction between biomolecules by electrochemical methods.

ACKNOWLEDGEMENTS
We are grateful to the National Natural Science Foundation of China (Nos. 20904066 and 21076232), Natural Science Foundation of Hunan Province, China (No. 10JJ6022) and Hunan Province scientific innovation projects, China（No.2012TT2050）for support.

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