# Analytical Methods

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Critical Review

# Molecular Imprinting Electrochemical Sensor for Selective Determination of Oxidized Glutathione

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**Abstract:** By using oxidized glutathione as a template molecule and *o*-Phenylenediamine as functional monomer, a molecularly imprinted polymer film was prepared on the surface of glassy carbon electrode via electro-polymerization. The recognition performance of molecularly imprinted membrane was characterized by cyclic voltammetry and electrochemical impedance spectroscopy. The detection for oxidized glutathione was conducted by differential pulse voltammetry based on the electrochemical reaction of potassium ferricyanide / potassium ferrocyanide on electrode getting through the cavities of molecularly imprinted membrane after the template molecules being eluted. The results showed that a linear relationship between oxidation currents and oxidized glutathione concentrations in the range of 0 ~ $8\times10^{-7}$ mol/L was observed with a detection limit of $1.8\times10^{-9}$ mol/L given a signal-to-noise ratio of 3:1. The sensor had been applied to assay of human plasma with satisfactory results.

**Keywords:** Molecular imprinting; Electrochemical sensor; Oxidized glutathione; Voltammetric method

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## 1. Introduction

Oxidized glutathione (GSSG), a form of glutathione, is an important active small peptide of an organism's antioxidant defense system. Glutathione has two forms, namely, reduced glutathione (GSH) and GSSG[1]. Under physiological conditions, GSSG and GSH in all tissue cells are in stable and constant dynamic equilibrium. In this equilibrium, GSSG has synergistic effect on GSH-removing radicals[2]. However, when the body is in a state of oxidative stress, this equilibrium is destroyed[3]. A variety of pathological damages, including neurodegenerative disease[4] and cardiovascular disease[5], can induce oxidative stress resulting to oxidation of GSH to GSSG, thereby damaging the equilibrium. Determination of GSSG in tissues enables us to understand oxidative stress of various target organs, and then prevent and treat the related diseases. Therefore, developing a rapid quantitative GSSG detection method in biological samples is important.

The current methods for GSSG determination are chromatography[6-7], capillary electrophoresis[8-9], fluorometric assay[10], electrochemical detection[11], chromatography-mass spectrometry[12] and enzyme assay[13]. These methods not only need expensive equipment, reagents, and complicated sample preparation, but also have other disadvantages, such as easy interference by sample components and poor enzyme stability.

Molecular imprinting refers to an experimental technique for synthesizing polymeric substances with specific target molecule recognition. This polymeric substance matches the target molecule's spatial structure and binding site[14].With in-depth development, molecular imprinting techniques have been widely used in biochemical separation[15], biosensors[16], drug analysis[17-18] and other fields because of its high specificity. Recently, applications in protein and amino acid separation and detection have gradually attracted attention[19]. However, molecular imprinting has not been employed to detect GSSG. *o*-Phenylenediamine (*o*-PD) is a common functional monomer. Poly-*o*-PD membrane formed via electro-polymerization has hydrophobic, hydrophilic, and alkaline functional groups and many other characteristics[20-21]. The membrane thickness and density formed via electro-polymerization can be controlled[22]. Therefore, using *o*-PD as an monomer in preparing molecular imprinting membranes has attracted wide attention and more studies[23-24].

In this study, *o*-PD and GSSG were used as functional monomer and template molecule, respectively, and electro-polymerization was used to prepare a new kind of sensor for detecting GSSG. The result showed that this kind of sensor was characterized by high sensitivity, simple preparation, and high recognition. Thus, it could be used in GSSG determination in biological samples.

## 2. Experiments

### 2.1 Reagents and instruments

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*o*-Phenylenediamine (*o*-PD), potassium ferricyanide ($K_3[Fe(CN)_6]$) and potassium ferrocyanide ($K_4[Fe(CN)_6]$) were purchased from Sinopharm Group Chemical Reagent Co., Ltd.; GSSG, GSH, and N-ethylmaleimide (NEM) were purchased from 5 Shanghai Xueman Bio-Tech Co., Ltd.; bovine serum albumin (BSA), the synthetic peptide I and II were bought from Shanghai Chutai Bio-Tech Co., Ltd. I was composed of peptide fragment ACG and ECG by disulfide bond; II was composed of peptide fragment ACA and ACA by disulfide bond.

10 All of the reagents were of analytical grade. Water used in the experiment was double-distilled. The CHI660C Electrochemical workstation was from Shanghai Chenhua Instruments Company. A three-electrode system was used, which was composed of a glassy carbon electrode (GCE) ($\Phi$ = 2 mm), platinum wire 15 electrode, and Ag/AgCl electrodes as the working, counter, and reference electrodes, respectively.

### 2.2 Plasma sample preparation

A total of 10 mL blood samples were extracted from three healthy male volunteers and placed in heparin pre-cooling tubes. 20 The plasma was separated after centrifugation at low temperature. To measure GSSG, 0.5 mL plasma was mixed with equal volume of 0.04 mol/L NEM and allowed to stand for 30 min (for stopping GSH in samples to transform into GSSG). The plasma samples were stored at low temperature in a refrigerator.

25 The written informed consent was obtained from all participants and all studies were approved by the Experimental Animal Research Committee of Guilin University of Technology and performed in accordance with National Institutes of Health guidelines.

### 30 2.3 Preparation of molecular imprinting polymer (MIP) modified electrodes

A glassy carbon electrode polished by 0.05 μm slurry of alumina powder was placed in 10 mL of acetate buffer solution (pH = 5.4, 25 °C) containing $6.66\times10^{-3}$ mol/L GSSG and $6\times10^{-3}$ mol/L *o*-35 PD. Cyclic voltammetry (CV) was used to make 30 scanning cycles in the potential range between 0.0 and +0.8 V at 0.05 V/s scanning speed. After electro-polymerization, the water–alcohol solution (50%, V/V) was used to remove template molecules so as to prepare a molecular imprinting membrane with imprinted 40 cavities. As a control, a non-molecular imprinting polylmer (nMIP) modified electrode was also prepared under the same experimental conditions in the absence of GSSG.

### 2.4 Electrochemical measurement

Electrochemical measurement was performed using $3\times10^{-4}$ mol/L 45 potassium ferricyanide / potassium ferrocyanide solution containing 0.5 mol/L potassium chloride to characterize molecular imprinting membrane. CV and differential pulse voltammetry (DPV) were performed from -0.2 V to +0.7 V at a scan rate of 50 mV/s. Electrochemical impedance spectroscopy 50 (EIS) was performed at 25 ºC on an AutoLab PGSTAT302 (Eco Chemie, Utrecht, The Netherlands).

## 3. Results and discussion

### 3.1 Performance of the molecular imprinting membrane

55 Under the given experimental conditions, the redox peaks of probe were recorded in the −0.2 V to +0.7 V range. Potassium ferricyanide had better oxidation reduction peak because of relatively small size and ease of passage through imprinted cavities to react at electrode surface. Therefore, potassium 60 ferricyanide could be used as probe between the imprinting membrane electrode and substrate solution to characterize molecular imprinting membrane recognition performance. The result was shown in Fig. 1, in which a referred to bare electrode and b referred to electrode after deposition of polymer film. The 65 probe did not reach the electrode surface, thereby inhibiting the occurrence of a redox reaction, which resulted in current reduction. The process from b to c referred to template removal. After GSSG removal, the probe reacted on the imprinting membrane electrode from the exposed cavities, thus, the current 70 increased. And after template molecule rebinding occurred from c to d, the special recognition site was re-occupied resulting to a smaller current than the removal current. In contrast, for nMIP modified GCE, the peak current was dramatically decreased from curve e to curve f in Fig.1B, illustrating the formation of non-75 conductive membrane in the absence of template molecules. The current remained unchanged after the removal step (curve g) due to the lack of cavities with binding sites. It demonstrated that the nMIP-GCE failed to recognize GSSG.

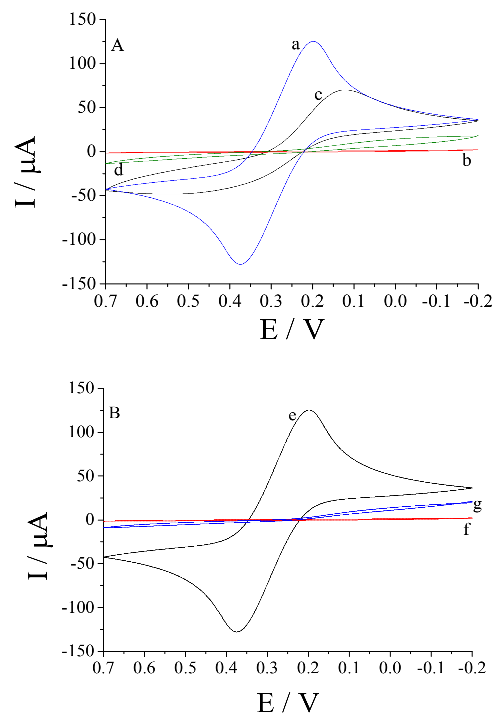


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**Fig. 1** CV curves of the MIP-GCE under different conditions: (a,

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e) bare glassy carbon electrode; (b) MIP-GCE; (c) MIP-GCE after template removal; (d) MIP-GCE after rebinding; (f) nMIP-GCE; (g) nMIP-GCE after the removal of template

### 3.2 Alternating current impedance measurement

Alternating current (AC) impedances were measured to confirm that the MIP modified electrodes were properly produced. Fig. 2 showed the changes in the MIP formation and template elution and rebinding. The increased resistance from curve a to curve b could be attributed to the produced MIP film that covered the surface of GCE. The decrease of resistance from curve b to curve c could be attributed to the removal of GSSG from the MIP. The increased resistance from curve c to curve d signified that GSSG was rebound to the film. This verified that the MIP film has good capability to distinguish the target molecule.

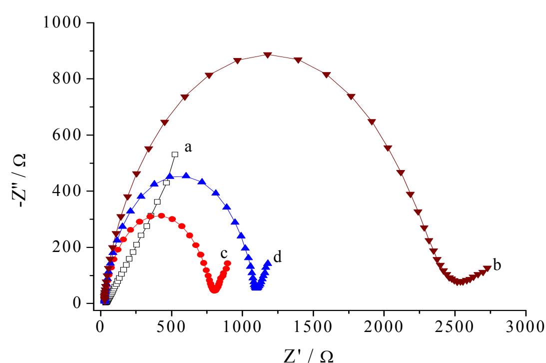


Fig.2 AC impedances of MIP films in different conditions
a. GCE; b. MIP modified GCE; c. MIP-GCE after GSSG removal; d. MIP-GCE after rebinding with GSSG

### 3.3 Optimization of the analytical parameters

The functional monomer to template molecule ratio is an important factor influencing the response current of a sensor. DPV was employed for the current measurements, which was relatively sensitive compared to the conventional CV method. The response current was highest when the molar ratio of *o*-PD to GSSG reached 3:1 as shown in Fig. 3a. With the decrease in this ratio, response current also decreased. Therefore, the ratio was 3:1.

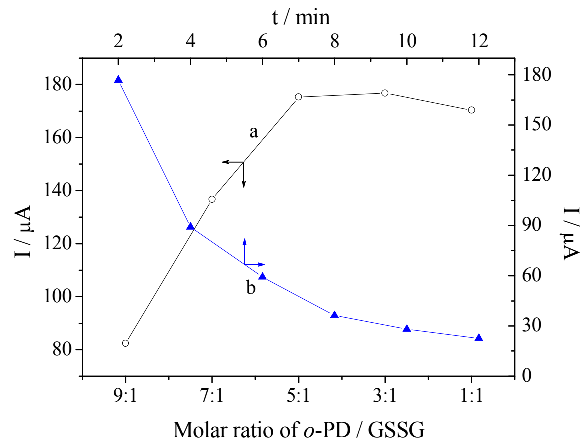


**Fig. 3** Effect of the molar ratio of *o*-PD/GSSG(a) and rebinding time (b) on DPV peak current

To obtain the optimal incubation time, the MIP modified electrodes were immersed in GSSG solution and then the DPV curves were recorded in potassium ferricyanide every 2 min. The relationships between the peak current (I) of DPV and the rebinding time (t) were checked. As shown in Fig. 3b, with the increasing of rebinding time, peak current gradually decreased. After reaching 12 min, the current remained unchanged due to the saturation of the binding sites in the MIP membrane. Therefore, rebinding time was set to 12 min.

### 3.4 Calibration curve

Under optimal conditions, DPV was used to detect the content of GSSG after MIP modified electrode was placed in GSSG solution in different concentrations. As shown in Fig. 4, with the increase in GSSG concentration, oxidation peak current gradually decreased and the linear relationship between peak current (I) and GSSG concentration (C) in the 0 to $8\times10^{-7}$ mol/L range was improved. The linear regression equation was I (μA) = 179.302 - 2.1014C ($10^{-8}$ mol/L). The regression equation revealed a good linear relationship ($R^2$=0.996) within the test ranges. The detection limit was $1.8\times10^{-9}$ mol/L, which was lower than that reported in other methods[6-13].

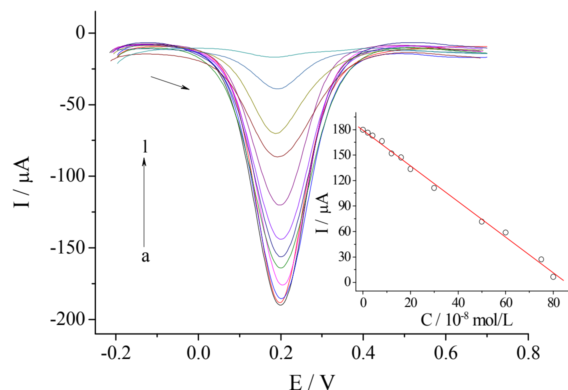


**Fig. 4** DPV of MIP-GCE after incubation in different GSSG concentrations



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a→l: 0, 2, 4, 8, 12, 16, 20, 30, 50, 60, 75, $80\times10^{-8}$ mol/L GSSG, respectively

### 3.5 Selectivity, repeatability, and stability of sensor

After template removal, MIP modified electrodes were immersed in $7\times10^{-7}$ mol/L GSSG, $1.5\times10^{-5}$ mol/L peptide I, $2\times10^{-5}$ mol/L peptide II, $5\times10^{-5}$ mol/L GSH, $3\times10^{-5}$ mol/L ascorbic acid, $3.5\times10^{-5}$ mol/L glutamic acid, $6\times10^{-5}$ mol/L cysteine and $8\times10^{-5}$ mol/L BSA. The electrodes were washed after 12 min incubation and placed in potassium ferricyanide solution for DPV scanning. The peak currents generated from various substances were obtained. The result was shown in Fig. 5. The MIP sensor had specific GSSG selectivity, weak specificity to other structural analogs, and almost no protein interference, proving that this sensor had good GSSG selectivity.

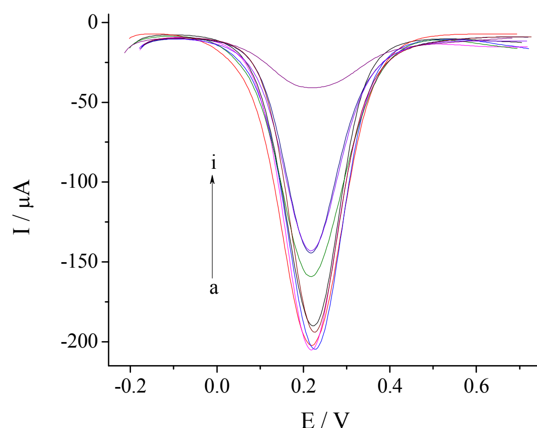


**Fig. 5** DPVs of MIP-GCE in different conditions

a. after template removal; b. after rebinding BSA; c. after rebinding ascorbic acid; d. after rebinding cysteine; e. after rebinding glutamic acid; f. after rebinding peptide II; g. after rebinding peptide I; h. after rebinding GSH; i. after rebinding GSSG.

Upon optimization, this sensor detected $1\times10^{-7}$ mol/L GSSG for nine times. After each measurement, the electrode was washed with 50% ethanol for 5 min to remove the template molecules. Its relative standard deviation (RSD) was 2.1%, indicating that this kind of sensor had good repeatability. Meanwhile, five times of parallel determination were conducted every other day. Peak current was reduced to 5.6% within 10 d, denoting good sensor stability.

## 4. GSSG determination in plasma samples

The sensor was placed in a treated sample for rebinding and determination of current response signal. The sample could be gradually diluted until tested. Standard recovery test was performed at the same time. The results were shown in Table 1. The recovery rate of the proposed molecular imprinting electrochemical sensor was in the range of 98.7% to 100.3%. The RSD was lower than 3%, proving that this sensor had good recovery and applicability.

Table 1. **Results of plasma sample anasysis(n=5)**

| Samples | Found (μmol/L) | Added (μmol/L) | Total found (μmol/L) | RSD (%) | Recovery (%) |
|---|---|---|---|---|---|
| 1 | 1.21 | 3 | 4.17 | 2.61 | 98.7 |
| 2 | 1.37 | 6 | 7.39 | 1.68 | 100.3 |
| 3 | 1.43 | 10 | 11.39 | 1.53 | 99.6 |

## 5. Conclusion

A highly sensitive and selective MIP sensor for GSSG detection was successfully developed by measurement of the oxidative current of potassium ferricyanide, an electrochemical probe, getting through the cavities of MIP after the template molecules being eluted. The detection limit of the proposed sensor was lower than previously reported methods. GSSG in plasma samples was successfully determined by the present MIP sensor. It could be a useful method for medical diagnosis, practical analyses are recommended.

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# Graphic Abstract

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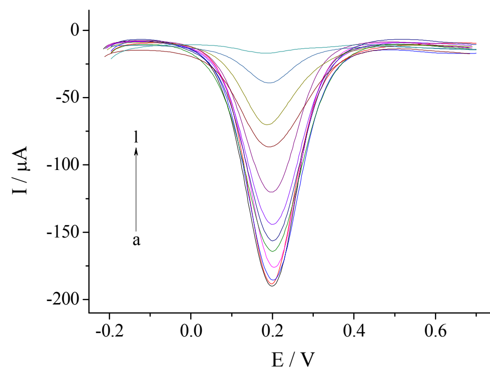


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