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# Biosensing approach for glutathione detection using glutathione reductase and sulfhydryl oxidase bienzymatic system

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**Abstract**

Chitosan membrane with glutathione reductase and sulfhydryl oxidase (SOX) was subsequently integrated onto the surface of spectrographic graphite rods for obtaining a glutathione biosensor. The working principle was based on the monitoring of $O_2$ consumption that correlates the concentration of glutathione during the enzymatic reaction. A linear relationship between sensor response and concentration was obtained between 0.5 and 2.0 mM for oxidized glutathione (GSSG), and 0.2–1.0 mM for reduced glutathione (GSH) in the presence of 2 μM nicotinamide adenine dinucleotide phosphate (NADPH) under the optimum working conditions. Also, reduced/oxidized glutathione were separated by HPLC and utility of bienzymatic system was investigated as an electrochemical detector for the analysis of these compounds. All data were given as a comparison of two systems: biosensor and diode array detector (DAD).


*Keywords:* Glutathione reductase; Sulfhydryl oxidase; Reduced/oxidized glutathione; Electrochemical detection

## 1. Introduction

Thiolic compounds are characterized by the presence of sulfhydryl residues [1]. The biological thiols can by classified as high-molecular mass protein thiols and low-molecular mass free thiols. As well as disulfides, low-molecular weight thiols such as glutathione (GSH), cysteine (Cys), and homocysteine (HCys), have numerous roles in metabolism and homeostasis [2–4].

Glutathione is widely found in all forms of life and plays an essential role in the health of organisms, particularly aerobic organisms. In animals, including humans, and in plants, glutathione is the predominant non-protein thiol that functions as a redox buffer keeping with its own SH groups those of proteins in a reduced condition, among other antioxidant activities [5–8]. Reduced glutathione (GSH), a tripeptide (γ-glutamylcysteinylglycine) with a free thiol group, is a major antioxidant in human tissues. It provides reducing equivalents to the reactions of the glutathione peroxidase (GPx) catalyzed reduction of hydrogen peroxide and lipid hydroperoxides to water and the respective alcohol. During this process GSH becomes oxidized glutathione (GSSG). The GSSG is then recycled into GSH by glutathione reductase (GR) and nicotinamide adenine dinucleotide phosphate (NADPH) [9–11]. When mammalian cells are exposed to oxidative stress, the ratio of GSH/GSSG will reduce as a consequence of GSSG accumulation. Altered levels of glutathione in plasma have been implicated in a number of pathological conditions, including Alzheimer's, Parkinson's diseases, diabetes, mascular degeneration, and HIV disease [12–16]. Due to the importance and wide spread existence of glutathione and the possible use of its plasma levels as a biomarker of health status in many crucial biological functions, measurement of the GSSG level or determination of the GSH/GSSG ratio becomes a useful indicator of oxidative stress. Besides it can be used to monitor the effectiveness of antioxidant intervention strategies [17–19] and also its measurement is considered a primary goal in clinical chemistry [20].

A composite sulfhydryl oxidase (SOX) electrode was previously developed by our group and applied as an electrochemical biodetector for reduced thiolic compounds after HPLC separation [21]. In this work, an amperometric biosensor by co-immobilization of glutathione reductase (GR) and sulfhydryl oxidase by means of chitosan membrane was described. After

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optimization of the working conditions and examination of analytical characteristics, proposed system was applied for GSH and GSSG analysis in blood samples. The objective of this study was also to combine biocatalytic detection with HPLC separation for subsequent detection of reduced and oxidized forms of glutathione in the same matrices.

Glutathione reductase GR (EC 1.6.4.2, NADPH: oxidized glutathione oxidoreductase) is a ubiquitous enzyme, which catalyzes the reduction of oxidized glutathione (GSSG) to glutathione (GSH) [22]. Besides, SOX, an oxidoreductase with a cofactor of FAD, could oxidize thiols through the oxidization of sulfhydryl residue [23–26]. When two enzymes were co-immobilized, bienzymatic reaction that occurred in the bioactive layer was given below;

$$GSSG + NADPH + H^+ \xrightarrow{GR} 2GSH + NADP^+$$

$$G\text{–}SH + HS\text{–}G + O_2 \xrightarrow{SOX} R\text{–}S\text{–}S\text{–}R + H_2O_2$$

The proposed biosensor was based on the monitoring of consumed $O_2$ that correlates the concentration of glutathione as a result of the first enzymatic reaction which was shown above.

## 2. Experimental

### 2.1. Material

Sulfhydryl oxidase (SOX; EC 1.8.3.2, 2.0 U/ml, 0.4 mg protein/ml) was obtained X-Zyme GmbH (Düsseldorf, DE) and glutathione reductase (GR; Type II, EC1.6.4.2, 0.088 U/mg protein) from wheat germ, glutathione (GSH; reduced and GSSG; oxidized form), β-nicotinamide adenine dinucleotide phosphate, reduced form (β-NADPH), glutaraldehyde, chitosan, were purchased from Sigma (St. Louis, MO, USA). All other chemicals were analytical grade. All solutions were prepared with water purified with a Milli-Q (Millipore, Bedford, MA, USA) system.

1.0 wt.% chitosan solution was prepared by dissolving 1.0 g of chitosan flakes into 100 ml of 1.0% acetic acid and stirred for 3 h at room temperature until complete dissolution [27]. The chitosan solution was stored in refrigerator when not in use.

Blood samples were obtained from a local hospital and prior to use, samples were centrifuged. And then, buffy-coat was denaturized by adding 70% $HClO_4$. Finally, the obtained sample was diluted 10-fold and spiked with known amount of standard glutathione solution.

### 2.2. Apparatus

Chronoamperometric experiments were performed by a Radiometer electrochemical measurement system (VoltaLabPGP201) and bienzyme electrode was used as a working electrode. Ag|AgCl (with 3 M KCl with saturated AgCl as an internal solution, Radiometer Analytical, REF321) and Pt (Radiometer Analytical, M241PT) were used as reference and counter electrodes respectively. The electrodes were inserted into thermostatic reaction cell (25 ml).

High-performance liquid chromatography (HP1100) with a photodiode array detector (DAD) and controlled by a HP-Chemstation from Agilent (Karlsruhe, Germany).

### 2.3. Preparation of biosensors

For the construction of the bienzyme layer on the top of the graphite electrode, first the spectrographic graphite rods (Ringsdorff Werke GmbH, Bonn, Germany, 3.05 mm diameter and 13% porosity) were polished on wet emery to obtain a smooth surface, washed thoroughly with distilled water and sonicated for 2 min, and then rinsed again with water and dried in an oven at 105 °C [28].

Premixed solution composed of 20 μl chitosan solution, 10 μl GR (0.002 U) and 10 μl SOX (0.02 U) were placed on top of the polished end of the electrode and spread evenly. Bienzyme electrodes were kept at 4 °C overnight and treated with the glutaraldehyde solution (1.0% in phosphate buffer) for 30 min at 4 °C [29], then washed with distilled water.

### 2.4. Measurements

Measurements were carried out in phosphate buffer (50 mM, pH 6.5) medium containing coenzyme, NADPH, under the operating potential of −0.7 V by monitoring of consumed $O_2$ while the solution was stirred. The current changes were registered in terms of current density (nA/cm$^2$ or μA/cm$^2$) in 1 min of response time. After each measurement, the electrode was rinsed with distilled water and allowed to equilibrate in working buffer before another measurement. During the experiments, daily prepared electrodes were used.

In order to examine the non-enzymatic reaction of GSH, GSSG substrates as well as NADPH at the electrode surface, blank measurements were performed for each compounds and no interference was observed.

### 2.5. Chromatographic system and conditions

Separation was carried out by HPLC with DAD. The flow rate was 1.4 ml/min and detection wavelength was set at 220 nm. Reduced/oxidized glutathione were passed through a Waters Spherisorb ODS-2 $C_{18}$ column (250 mm × 4.6 mm, 5 μm) and the column temperature was 37 °C. The mobile phase consisted of 3.0% acetonitrile, 0.1% TFA (v/v) using an isocratic elution [30].

When bienzyme electrodes were used as an electrochemical detector for GSH and GSSG separation, these compounds that passed through the $C_{18}$ column were transferred by tubing system into the biosensor reaction cell containing working buffer solution as well as coenzyme afterwards, electrode responses at the working potential were registered as current densities. The retention times of both substrates (GSH, GSSG) were primarily estimated by DAD and then used to analyze the mixture of oxidized and reduced forms of glutathione by the proposed biosensing system.

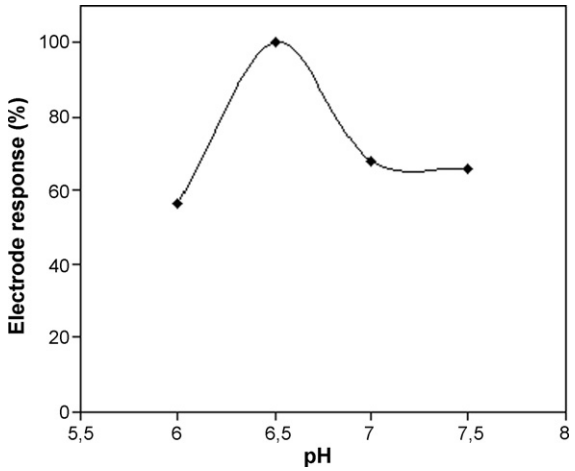


Fig. 1. Effect of pH on the electrode response for GSSG (in 50 mM phosphate buffers (pH 6.0–7.5) at 30 °C; −0.7 V; GSSG: 0.5 mM; NADPH: 2 μM).

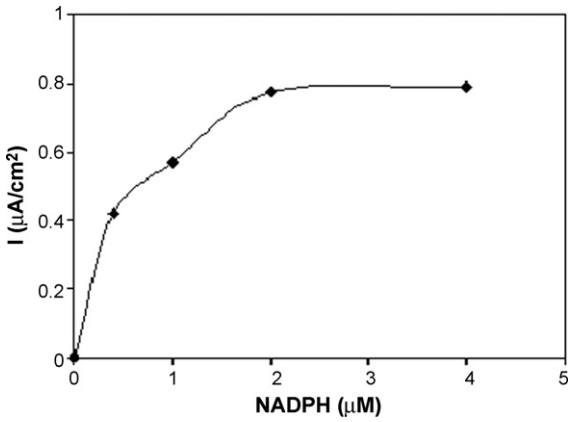


Fig. 2. Effect of NADPH on the electrode response for GSSG (in phosphate buffer; 50 mM at pH 6.5; 30 °C; −0.7 V; GSSG: 0.5 mM).

## 3. Results and Discussion

Glutathione measurement has always been proven to be difficult. Most of the methods are time consuming and either needs derivatization or they are lack of adequate sensitivity [31–36]. Compared to spectrophotometric methods, electroanalysis has the advantages of simplicity and high sensitivity and also the possibility of being used with colored samples without applying any pre-treatment. A less labor-intensive way for thiol measurement is the sensor method. A variety of amperometric systems for thiol detection has been previously described [37–42]. These systems generally do not require extensive sample preparation and have fast response [43,44].

The optimized and characterized biosensor was applied to online detection of reduced (GSH) and oxidized glutathione (GSSG) after HPLC separation. As mentioned before, when a composite electrode containing only SOX enzyme was used, only reduced thiolic compounds such as GSH could be analyzed [21]. Combination of GR and SOX enzyme enable us to detect GSSG as well as GSH in the same matrices.

### 3.1. Optimization of biosensor response

#### 3.1.1. pH effect

The effect of pH on the bienzyme electrode was investigated by using phosphate buffer at 50 mM between pH 6.5 and 7.5 (Fig. 1). The response of the bienzyme electrode increases significantly from pH 6.0 to 6.5, and then a decrease is obtained at higher pH values. As a result pH 6.5 was chosen as optimum pH and used for further studies.

#### 3.1.2. NADPH amount

To examine this effect, the responses of bienzyme electrodes were measured in the presence of different amounts of NADPH (0.5–4.0 μM) and plotted versus coenzyme concentration (Fig. 2). The best result was obtained with 2.0 μM of NADPH, higher concentrations caused the same response so further experiments were carried out by using this value.

### 3.2. Analytical characteristics

#### 3.2.1. Linear range

Linearity was obtained in concentration range between 0.5 and 2.0 mM of GSSG in the presence of NADPH (2.0 μM). As well as GSSG, the response for GSH which is a substrate of SOX enzyme that takes place as a constituent of bienzyme layer was also investigated and found in the range of 0.2 and 1.0 mM, respectively. At higher concentrations, standard curve showed a deviation from linearity (Fig. 3).

Repeatability was tested by the subsequent measurements ($n = 5$) by bienzyme electrode standard deviation (S.D) as well as variation coefficient (c.v) were calculated as $1.082 \pm 0.011$ mM and 1.0% for 1 mM GSSG while these values were found as $0.422 \pm 0.008$ mM and 1.9% respectively for 0.4 mM GSH.

The stability of the biosensor was investigated at working conditions (30 °C in phosphate buffer) and 11% decrease in the activity was observed after 3 h. During this period approximately 20 measurements were made.

### 3.3. Chromatographic separation

GR has substrate specificity mainly to GSSG in contrast to the SOX enzyme which acts different thiolic compounds in reduced forms such as GSH [22]. It is well known that thiolic compounds were found together in different matrices particularly in physiological fluids, plant extracts as well. Chromatographic separation of these components is therefore a necessary requirement for an accurate analysis of each substrate. Especially, measurement of GSSG level or determination of the GSH/GSSG ratio is very important as a marker of oxidative stress [45], as mentioned before. It is known that the accurate measurement of GSSG levels is very difficult due to the low GSSG amount in tissues. In this study, the proposed bienzyme electrodes were initially, characterized and then combined with HPLC system as a post column electrochemical detector to analyze GSSG in the presence of GSH. At the beginning, as we have done in our previous work [21], DAD was utilized to obtain retention times of GSH and GSSG respectively. Additionally, the calibration graphs for both substrates were also drawn by using DAD. As a result, sim-

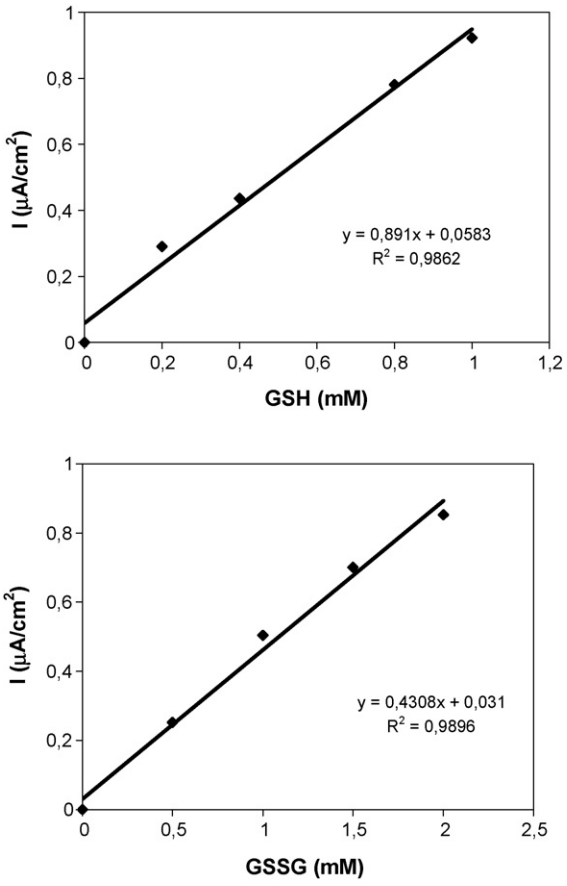


Fig. 3. Calibration graph for the GSH and GSSG determination (in phosphate buffer; 50 mM, pH 6.5, 30 °C, −0.7 V; NADPH: 2 μM).

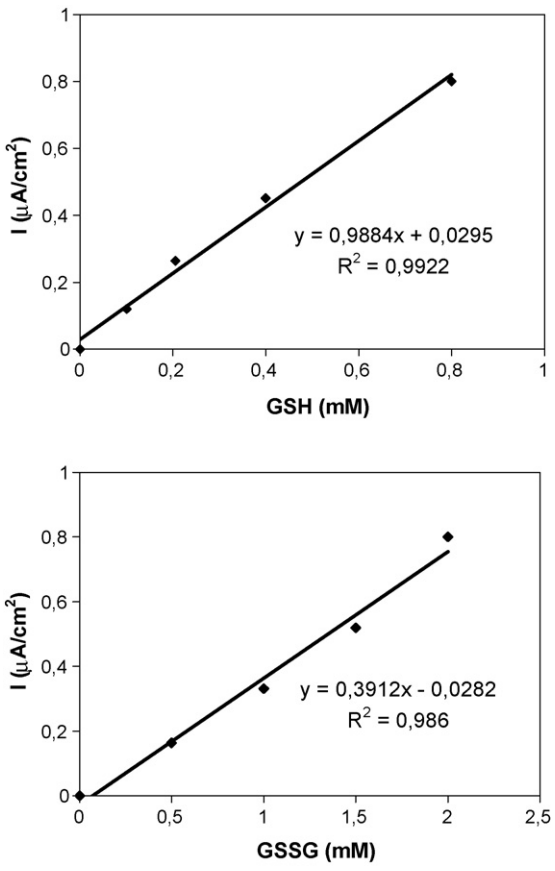


Fig. 4. Detection of GSH and GSSG after HPLC separation by GR-SOX biosensor as an electrochemical detector (−0.7 V; NADPH: 2 μM).

ilar data were obtained in terms of linearity (0.25 and 1.5 mM for GSSG and 0.125 and 1.5 mM for GSH, respectively) and the equation of linear graphs with Ref. [21]. These calibration graphs were used in sample application to estimate the glutathione amounts in blood sample as a reference method.

Furthermore, calibration graphs were obtained for GSSG and GSH that were applied to HPLC and analyzed by bienzyme biosensor as a biodetector. In this case linearity was obtained in concentration range between 0.1 and 0.8 mM for GSH and between 0.5 and 2.0 mM for GSSG (Fig. 4).

Chromatograms obtained by DAD resulting from injection of a GSH and GSSG mixture were presented in Fig. 5. The flow rate of the mobile phase was optimized to yield both good chromatographic resolution and electrode responses. Lower flow rates caused peak broadening. A flow rate of 1.4 ml/min caused quite acceptable results with better peak symmetry. Running the system at higher flow rates did not exhibit further advance. Hence, a flow rate of 1.4 ml/min was chosen as the optimal flow rate.

### 3.4. Sample application

In order to observe the sample matrix effects for glutathione analysis, standard addition method was used. Denaturized blood sample was prepared as described in Section 2 and used as stock solution. Initially, samples were applied to HPLC under the optimized conditions and analyzed by both DAD and biosensing system. DAD was used as a reference to evaluate these findings. In case of using biosensor system, when the samples passed through the column and reached to the reaction cell, changes in the response were recorded.

Finally, obtained signals from both DAD and biosensor systems were converted to the concentration values via GSH and GSSG calibration curves. Data for blank (non-spiked) and spiked samples were compared in terms of recoveries (Table 1). As shown in the table, recovery values were very similar to 100%. As a result, it can be concluded that the nature of sample does not affect the measurements. As a result developed

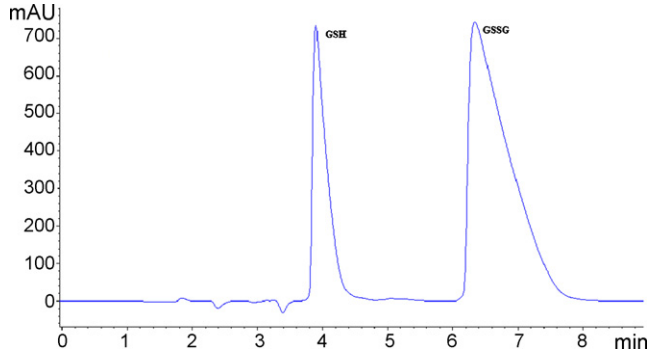


Fig. 5. Chromatogram of standard mixed reduced/oxidized glutathione [GSH and GSSG: 1.5 mM]. Chromatographic separation was performed according to the conditions described in Section 2.5.

Table 1
Application of glutathione biosensor to human blood sample after HPLC separation

| | Amount of glutathione in sample (mM)[a] ($C_0$) | Added glutathione (mM)[a] ($C_{add}$) | Detected glutathione (mM)[a] ($C_{tot}$) | % Recovery $(C_{tot} - C_0)/C_{add}$ |
|---|---|---|---|---|
| DAD[b] | | | | |
| GSH | 0.156 | 1.00 | 1.10 ± 0.06 | 94 |
| GSSG | 0.013 | 0.266 | 0.271 ± 0.0034 | 97 |
| GR-SOX biosensor | | | | |
| GSH | N.D | 0.20 | 0.21 ± 0.014 | 105 |
| GSSG | N.D | 0.50 | 0.50 ± 0.0282 | 100 |

N.D: Not detected. All results are given as value ± S.D, $n = 3$ or 4.

[a] Glutathione concentration were calculated using the standard curves obtained for each substrate.

[b] For the detection by DAD system, calibration graphs with the following equations were used for GSH and GSSG analysis ($y = 6921.3x$ and $y = 25460x$, $x$ and $y$ shows substrate concentration, mM and peak area, mAus, respectively) [21].

biosensor shows promising results to be used in the analysis of glutathione in both oxidized and reduced forms without requiring any laborious derivatization steps.

## 4. Conclusion

An amperometric biosensor based on the immobilization of two enzymes is developed both for GSSG and GSH detection. Glutathione reductase and sulfhydryl oxidase were immobilized onto a graphite electrode and the change in current density due to the oxygen consumption was monitored. The obtained data confirm the most attractive property of the new system which is the possibility to detect subsequently two different substrates without application of any complex treatment procedure prior to the measuring step. Hence they proved that the proposed biosensor is a simple, practical and sensitive tool for GSSG and GSH analysis. Because of these attractive properties, developed bi-enzymatic electrode in combination with HPLC separation can be accepted as an alternative method for GSSG and GSH detection.

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