



Anal. Bioanal. Chem. Res., Vol. 5, No. 1, 115-129, June 2018.

Electrocatalytic Determination of Glutathione Using Transition Metal Hexacyanoferrates (MHCFs) of Copper and Cobalt Electrode Posited on Graphene Oxide Nanosheets

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(Received 20 August 2017, Accepted 14 January 2018)

A glassy carbon electrode was modified with graphene oxide nanosheets and a hybrid of copper-cobalt hexacyanoferrate. The electrode surface was characterized by scanning electron microscopy. Cyclic voltammetry showed a stable and reversible redox pair with surface confined characteristics in phosphate buffer solution (pH 3). The prepared electrode showed electrocatalytic properties in the heterogeneous electron transfer process of glutathione. Hydrodynamic amperometry was used for determination of glutathione at pH 3. The catalytic oxidation peak current varied linearly with the concentration of glutathione in the range of 3.3×10^{-7} - 5.5×10^{-5} M with a limit of detection of 2.5×10^{-7} M. The repeatability was evaluated for 5 successive measurements of glutathione (0.48 mM) by the proposed method as relative standard deviation (RSD%), which was 3.02%. When 3 different electrodes were used, RSD% for glutathione determination was 3.34%. Determination of glutathione in hemolysed erythrocyte samples by the proposed method was satisfactory, compared to the standard method.

Keywords: Glutathione, Graphene oxide, Copper-cobalt hexacyanoferrate, Hemolysed erythrocyte, Amperometry

INTRODUCTION

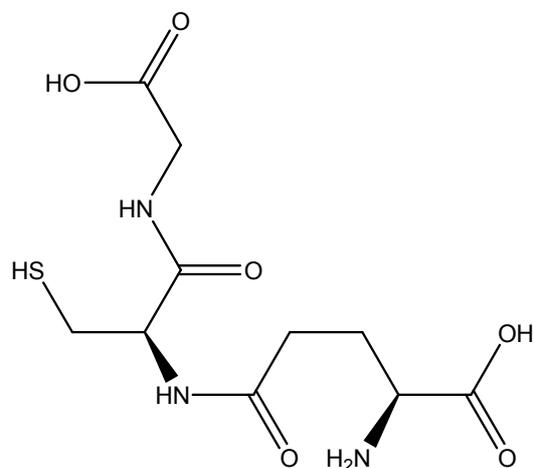
Glutathione (g-l-glutamyl-l-cysteinyl-glycine) (GSH) (Scheme 1) is a tri-peptide of glutamate, cysteine and glycine. More than 99.5% of GSH is localized in erythrocytes of human blood [1]. Change of GSH concentration in the biological fluids is a useful marker for detection of some diseases such as leukemia [2], diabetes [3], DNA-base damages [4] and in the investigation of some kinds of cancer [5]. Therefore, determination of GSH in human erythrocytes is potentially useful for clinical diagnosis of some diseases at the early stages.

A number of methods have been suggested for GSH analysis, such as HPLC with fluorimetric detection [6], amperometric detection on Pd-IrO₂ modified electrode [7],

and electrochemical detection [8], titration with tert-butyl hydroperoxide with end point detection by ¹H NMR [9], capillary zone electrophoresis [10], spectrophotometry [1, 11], spectrofluorimetry [12], flow injection analysis with chemiluminescence detection [13] and electrochemical methods [14-19]. Electrochemical methods have some unique and distinct advantages compared to other analytical methods, *e.g.*, rapidity, inexpensive instrumentation, high sensitivity and a simple operation procedure. The ability to modify electrode surfaces by various composites and nanomaterials has introduced an excellent superiority to electrochemical methods.

In the present work, electrodeposited transition metal hexacyanoferrates (MHCFs) on graphene oxide (GO) nanosheets was used for the amperometric determination of GSH. MHCFs are a group of interesting materials for electrode modification. They have received considerable

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Scheme 1. Chemical structure of glutathione

interest because of electron transfer ability during reduction and oxidation processes [20,21]. Flux of different ions is necessary for the charge balance during redox reactions. In Prussian blue (PB) due to the small diameter of channels this phenomenon is not possible but cobalt hexacyanoferrate (CoHCF) with an open and zeolite-like structure seem to be fairly open and permit transport of alkali-metal cations providing charge balance during redox reactions [22]. Modified electrode by CoHCF showed well-defined and reproducible electrochemical responses and has been used as detector for determination of glutathione in liquid chromatography [23], electrocatalytic oxidation of captopril [24,25], amperometric determination of morphine [26], hydrazine [27], dopamine, epinephrine, norepinephrine [28], paracetamol [29] and also for fabrication of biosensors [30]. Another PB analogue, copper hexacyanoferrate (CuHCF) is interesting in electroanalysis due to its remarkable electrocatalytic properties and capability of presenting reversible redox reactions [31]. CuHCF structure allows the different ions to flux into its zeolitic structure to balance charge, so, CuHCF presents an increased stability in physiological pH when compared to PB modified electrodes [32]. CuHCF has been used in amperometric determination of hydrogen peroxide [33], captopril [34], L-cysteine [35], glutathione [36], L-tryptophan [37], ascorbate [38] and in fabrication of biosensors [39]. One of the disadvantages of MHCs is their limited stability during operation in neutral and alkaline pHs [40]. A way to improve the stability of

MHCs is the use of hybrid metals instead of one metal center [41,42]. When two kinds of metal ions react simultaneously with $\text{Fe}(\text{CN})_6^{4-}$ or $\text{Fe}(\text{CN})_6^{3-}$, a hybrid MHC is formed, if the metal ions have similar radii, similar physicochemical properties and same lattice symmetry [43]. Hybrid copper-cobalt hexacyanoferrate (CuCoHCF) improves the stability of MHC film on the electrode surface and exhibits different electrochemical behavior compared to CuHCF and CoHCF [44]. The complex interactions between elements in the CuCoHCF decrease the hydrolysis of each element compared to that in CuHCF or CoHCF. Hybrid CuCoHCF was reported in electrochemical determination of mercury [43], electrocatalytic oxidation of thiols [45], captopril [46] and L-cysteine [47]. Due to the presence of cysteinyl moiety in GSH, it was supposed that CuCoHCF can be used for fabrication of a GSH-sensor.

GO nanosheets have been used as an excellent substrate for deposition of CuCoHCF hybrid on the surface of a glassy carbon electrode. GO is the oxygenated derivative of graphene (monolayer graphite). Because of the relatively large active surface area and the existence of oxygen-containing groups (hydroxyl, carboxyl, and so on), ionic species can be adsorbed on the surface of GO [48]. Due to the synergic electrocatalytic properties of MHCs/GO composite, its application has been expanded in electroanalysis [49].

In this work, GO nanosheets are used for deposition of

CuCoHCF hybrid on a glassy carbon electrode. Electrostatic attraction between the negative charges on GO and the metal cations induces a homogeneous deposition process that assists deposition of CuCoHCF with uniform shape and good dispersion on GO substrate. After surface characterization, the modified electrode is being successfully used in amperometric analysis of GSH.

EXPERIMENTAL

Apparatus

Electrochemical measurements were performed with μ -Autolab type III potentiostat/galvanostat instrument (Eco Chemie B.V, Utrecht, The Netherlands) and driven by the NOVA (Version 1.11) software in conjunction with a conventional three-electrode system and a personal computer for data storage and processing. The modified GCE was employed as the working electrode and a platinum wire as the counter electrode. All potentials were referred to a Ag/AgCl (KCl 3 M) electrode. All electrodes were from Azar electrode (Orumieh, Iran). The surface morphology and properties of the modified electrode were characterized by field emission scanning electron microscopy (FESEM Vega-Tescan). A pH meter (Jenway, Model 140) with a glass electrode was used to check the pH of the solutions.

Reagents and Solutions

GSH was purchased from Merck (Darmstadt, Germany). Stock solution of GSH (1.0×10^{-2} M) was daily prepared and stored in refrigerator at 4 °C. CuSO₄·5H₂O, CoCl₂, K₃Fe(CN)₆, KNO₃ and H₃PO₄ were in analytical grade from Merck (Darmstadt, Germany) and were used without further purification. In all electrochemical experiments, a stock phosphate buffer solution (PBS) was used as the supporting electrolyte. Buffer solutions of different pHs were prepared by the addition of 0.2 M sodium hydroxide. Healthy human blood serum samples were obtained from Imam Reza hospital (Kermanshah, Iran).

Graphene oxide (GO) was prepared using a modified Hummers method [50], starting from graphite powder. Briefly, graphite (3 g) was added to a solution contained sulfuric acid (360 ml) and phosphoric acid (40 ml) and stirred with a magnetic stirrer. Then, potassium permanganate (18 g) was added slowly to the mixture

during the stirring process. A solution of H₂O₂ was added to the mixture in an ice bath in the next step. The color of mixture changed to bright yellow, indicating a high oxidation level of graphite. The solution was filtered and washed several times with water to remove the remaining unreacted species. The washing process was carried out using a simple decantation of the supernatant with centrifugation at 5000 rpm for 30 min, which resulted in the formation of GO. The synthesized GO was dried in 60 °C for 24 h. FT-IR spectrum of the product confirmed the formation of GO (Fig. 1). Absorbance at the following frequencies were used in GO characterization [51]: O-H (3423 cm⁻¹); C=O (1735 cm⁻¹); C=C aromatic (1624 cm⁻¹); C-OH (1229 cm⁻¹); C-O (1092 cm⁻¹).

Preparation of the Modified Electrode

The glassy carbon electrode (GCE) was polished with emery paper followed by alumina (0.05 μ m), until a mirror like surface achieved which was thoroughly washed with distilled water. The GO suspension (1.0 mg ml⁻¹) was prepared by dispersing GO in double-distilled water with the aid of ultrasound bath, which was dropped on GCE surface (7 μ l) and dried at room temperature to obtain GO/GCE. It was then immersed in a freshly prepared solution containing KNO₃ (0.25 M), CuSO₄, CoCl₂ and K₃Fe(CN)₆, each 0.125 mM. Cyclic voltammetry was applied in a potential window from 0 to 1 V with scan rate of 50 mV s⁻¹ (15 cycles) for electrodeposition of CuCoHCF film [47] on GO/GCE. To increase reproducibility, in the current study, solutions were added in the order: KNO₃, CuSO₄, CoCl₂ and K₃Fe(CN)₆. After each experiment, the electrode surface was cleaned by repetitive cyclic voltammetry (5 cycles) in the potential range of 0.0 to +1.2 V (vs. Ag/AgCl, KCl 3 M).

Preparation of Real Samples

Human whole blood was obtained from Imam Reza hospital (Kermanshah, Iran) and erythrocytes were separated from whole blood by removing the plasma. The whole blood (2 ml) was centrifuged for 10 min at 3000 rpm. The supernatant (plasma) was discarded and the rest was mixed with 5 ml NaCl (0.9% w/v). The process of washing with NaCl solution was repeated three times in order to remove the plasma completely. The erythrocyte pellets were

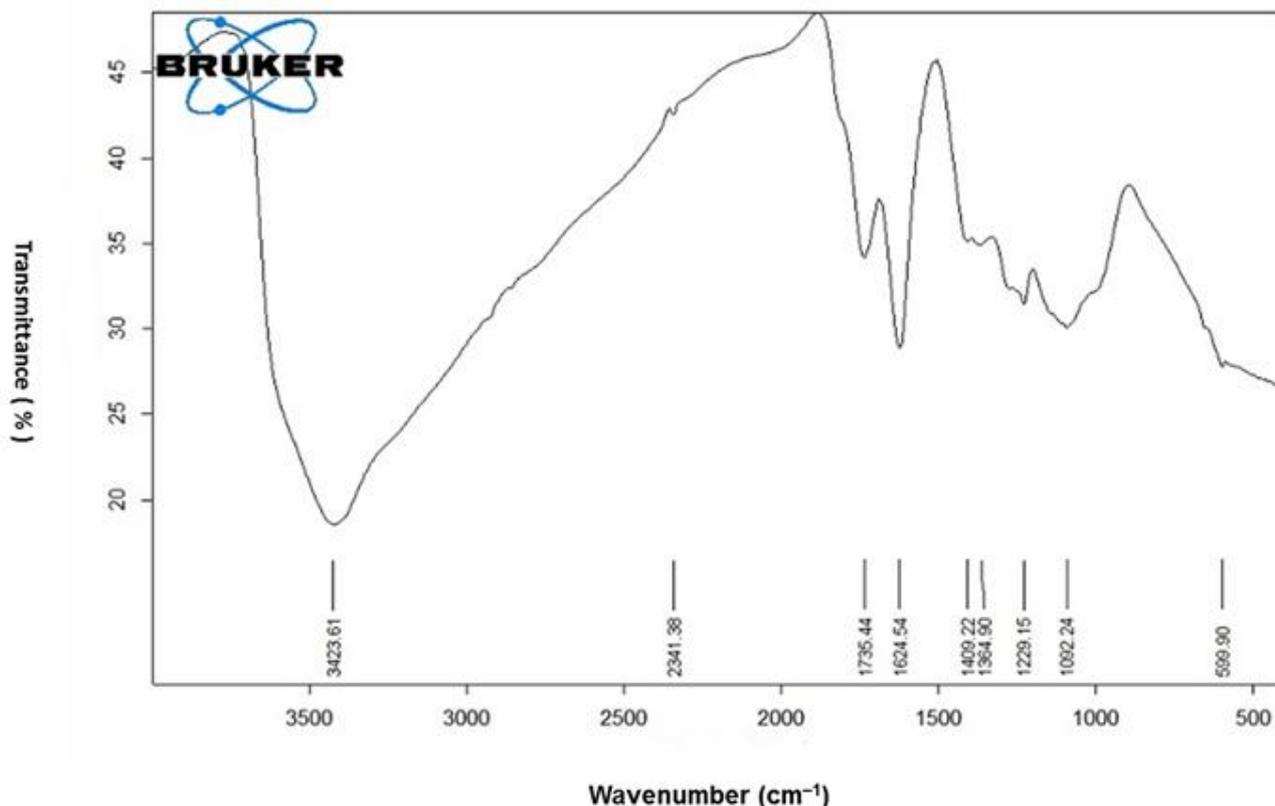


Fig. 1. FT-IR spectrum of the prepared GO.

hemolysed with water (1:1 v/v). For protein precipitation, the hemolysate erythrocyte was mixed with methanol (10% v/v) in the ratio 2:1 (v/v). The mixture was centrifuged. The supernatant was divided into two parts for spectrophotometric and electrochemical measurements. The Elman reference method which is based on the reaction of glutathione with 5,5'-dithio-bis-(2-nitrobenzoic acid, DTNB (Elman's reagent), generating 2-nitro-5-mercapto-benzoic acid [52] was carried out for spectrophotometric measurements. The product was monitored at 412 nm. The real samples were diluted (100 times) with PBS (0.1 M, pH 3) and were used for the recovery tests. Standard addition method was used for the determination of GSH in real samples. Although ascorbic acid and cysteine show interference, they are not present at significant levels in hemolysed erythrocyte samples [53]. In human blood, more than 99.5% of GSH is localized in erythrocyte, while 97% of cysteine is present in plasma [54].

RESULTS AND DISCUSSION

Electrodeposition of CuCoHCF on GO/GCE

MHCF are typically precipitated by electrodeposition (potentiostatic, galvanostatic, and cyclic voltammetry) on conductive substrates during a reduction process in a colloidal solution containing hexacyanoferrate(III), transition metal (M) salt and potassium or sodium electrolyte or *via* the generation of surface precipitates during oxidation (potentiostatic, galvanostatic) of a metal film which was previously deposited onto the substrate, in the presence of hexacyanoferrate (II) [55].

In this work, cyclic voltammetry (15 cycles) was applied to the surface of GO/GCE immersed in a solution containing KNO₃, CuSO₄, CoCl₂ and K₃Fe(CN)₆. Two redox couples (Fig. 2, I and II) were appeared and increased regularly during successive potential sweeps. This behavior indicated the formation of CuCoHCF on the electrode

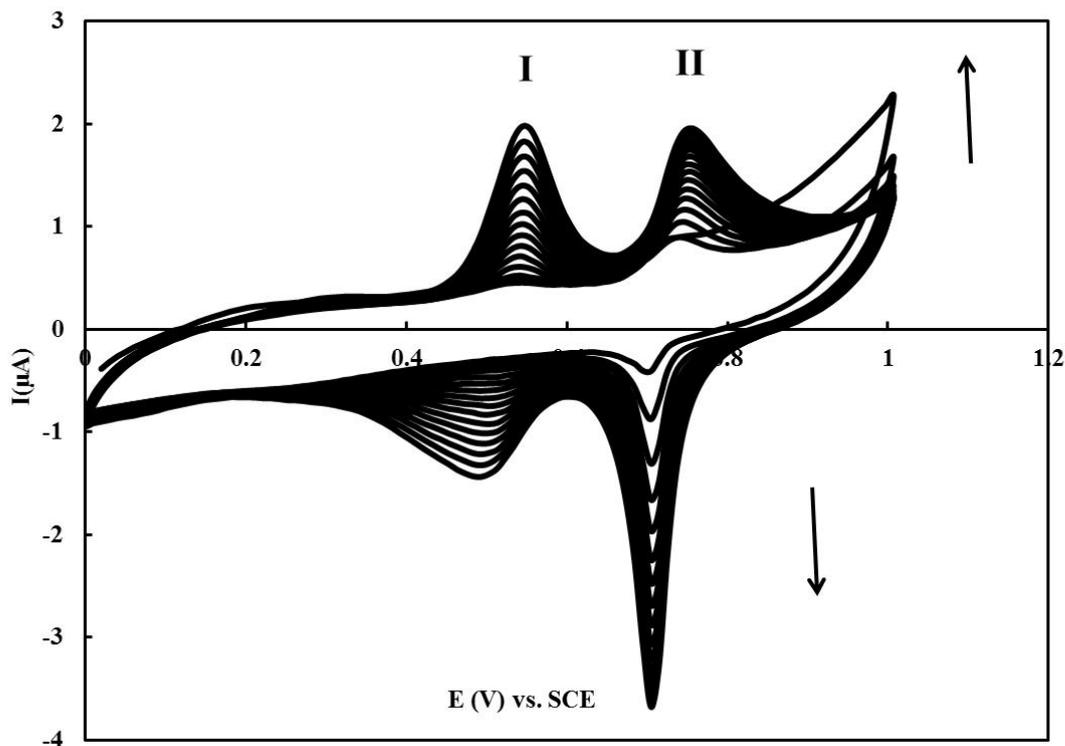


Fig. 2. Cyclic voltammetry in a solution containing KNO_3 (0.25 M), CuSO_4 (0.125 mM), CoCl_2 (0.125 mM) and $\text{K}_3\text{Fe}(\text{CN})_6$ (0.125 mM) at GO/GCE, scan rate of 50 mV s^{-1} .

surface [56]. Although different reasons were proposed for the appearance of two redox couples, it was attributed to electron transfer systems of $\text{Co}^{3+}/\text{Co}^{2+}$ (couple I) and $\text{Fe}^{3+}/\text{Fe}^{2+}$ (couple II) from more recent studies [25]. The formal potentials $E^{\circ'}$ were calculated as 0.523 and 0.725 V for couples I and II, respectively. The peak potential separation (ΔE_p) for I and II pairs was calculated to be 20 and 25 mV, respectively, indicating surface-confined moieties [57].

Surface Morphology of Modified Electrode CuCoHCF/GO/GCE

Modification steps were followed by scanning electron microscopy (SEM). GO layers are observed in Fig. 3 (left), while the spread of CuCoHCF nanocubes on GO layers are obvious in the right image. The high stability of the composite is probably due to the strong interaction between GO layer and CuCoHCF nanocubes. It was confirmed that

GO has reducing ability and is involved in the electron transfer of MHCF through redox reactions [49]. The results indicated that CuCoHCF keeps the usual properties of single component MHCFs, such as the dependence of peak potential of $\text{Fe}(\text{II})/\text{Fe}(\text{III})$ on the concentration of electrolyte ions.

Electrocatalytic Oxidation of GSH on CuCoHCF/GO/GCE

Electrochemical behavior of GSH was examined on the surface of CuCoHCF/GO/GCE by cyclic voltammetry (Fig. 4). A significant increase in anodic peak current of the modified electrode (couple II) was observed (curves a and b), while cathodic peak current decreased. The anodic and cathodic peak currents of couple I were almost unchanged in the presence of GSH. The direct oxidation of GSH at unmodified GCE showed no redox peak. The modified electrode showed excellent catalytic activity toward

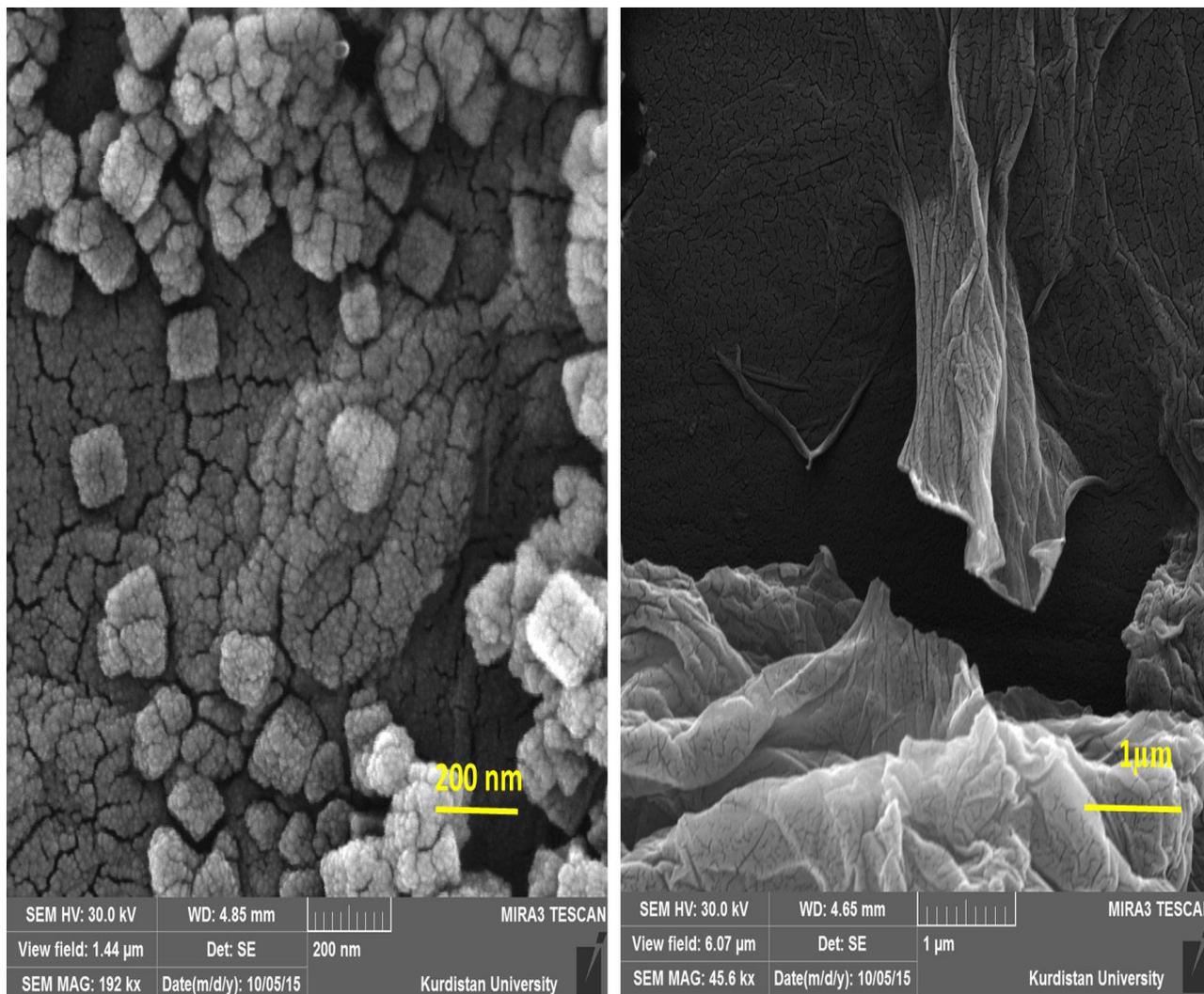


Fig. 3. Scanning electron microscopic (SEM) images of GO/GCE (right) and GO/CuCoHCF/GCE (left).

oxidation of GSH.

The following EC' mechanism (Scheme 2) was proposed based on the similarity between GSH behavior in this work and reported cyclic voltammograms of thiol compounds at the same modified electrode [45]:

The anodic peak appeared at 0.818 V corresponds to the oxidation of Fe^{2+} to Fe^{3+} in CoCuHCF/GO/GCE (step a in Scheme 2), which is greatly enhanced in the presence of GSH through a catalytic mechanism. GSH was oxidized to a radical GS^{\bullet} via a chemical reaction (step b) which was stabilized by dimerization (step c). The sulfur atom of GSH

(cysteine residue) was involved in electron transfer process. The charge balance of Scheme 2 is provided by contribution of stoichiometric amounts of Na^+ or K^+ .

Optimization of the Experimental Conditions Effect of pH

The effect of pH on the electrocatalytic oxidation of GSH at CoCuHCF/GO/GCE was investigated. The cyclic voltammograms of GSH solution (0.48 mM) were recorded at different pH values. In highly acidic solutions of GSH

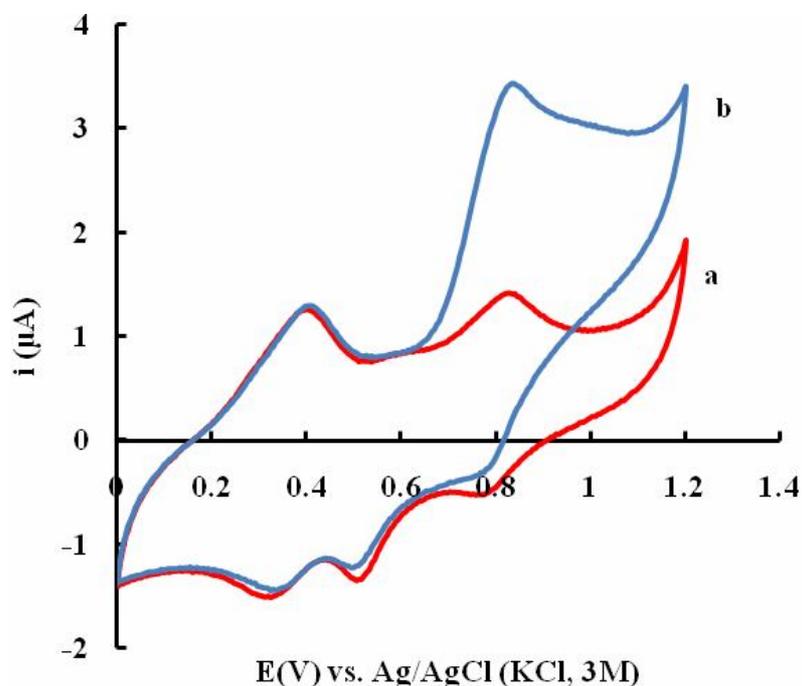
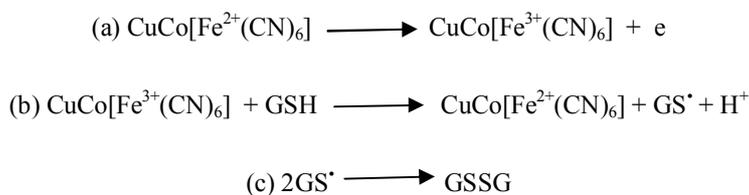


Fig. 4. Cyclic voltammograms of CoCuHCF/GO/GCE in the absence (A) and presence (B) of GSH (0.48 mM). Phosphate buffer solution (0.1 M, pH 3), scan rate 50 mV s^{-1} .



Scheme 2. EC' mechanism of GSH oxidation

(pH 3), the highly increased anodic current can be related to the involvement of H^+ ions in the electrooxidation mechanism of GSH (Scheme 2), as well as, the more stability of CuCoHCF film in acidic media (Fig. 5A). At pH > 4 , due to formation of Fe(OH)_3 at the modified electrode and its instability, large decrease in the magnitude of the catalytic current occurred [35]. Plotting anodic current against pH (Fig. 5B) shows the maximum current at pH 3. The peak potentials were almost unchanged at pH > 2 . Therefore, pH = 3 was selected as an optimum pH value for determination of GSH.

Effect of Scan Rate

The effect of scan rate (ν) on the electrocatalytic oxidation of GSH was studied at CuCoHCF /GO/GCE in the range of 10-100 mV s^{-1} (Fig. 6A). The anodic peak current increased linearly with square root of the scan rate (Fig. 6B), as for a diffusion current. Moreover, the plot of $\log(I_p^a)$ against $\log(\nu)$ was linear (Fig. 6C) with a slope of about 0.6, indicating the large contribution of diffusion current (slope of 0.5 is an indication of diffusional current, while that of unity is due to purely adsorptive current, $0.5 < \text{slope} < 1$ shows partial contribution of diffusion and

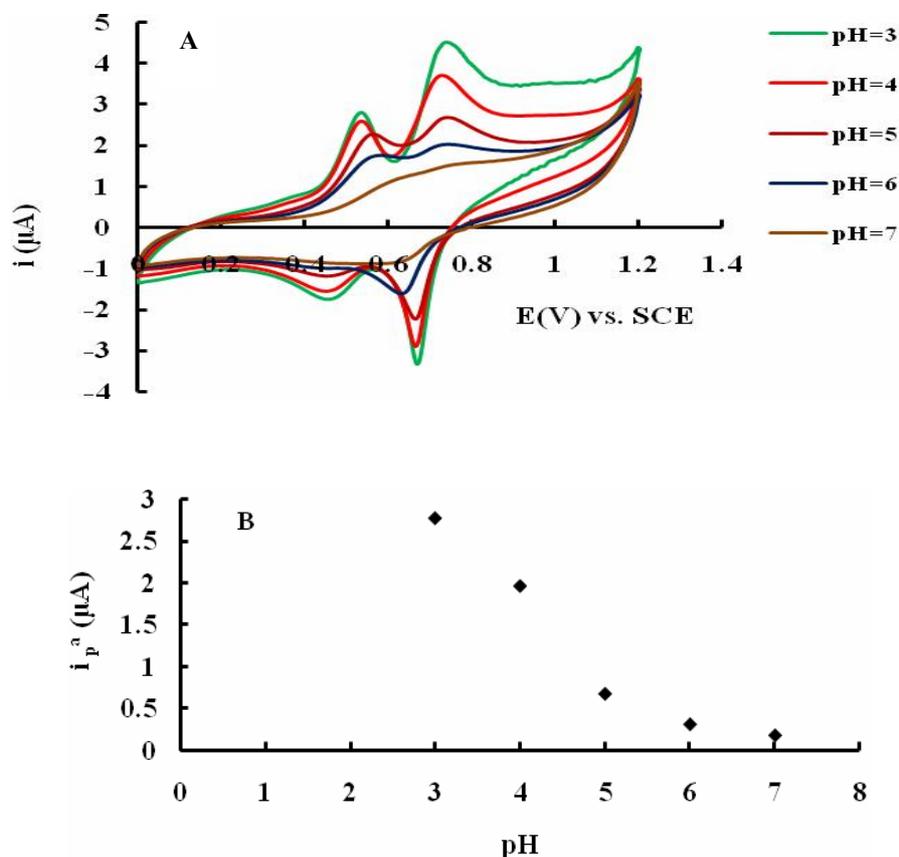


Fig. 5. (A) Effect of pH on cyclic voltammograms of CuCoHCF/GO/GCE in the presence of GSH (0.48 mM); (B) Plot of anodic peak current against pH. Scan rate: 50 mV s⁻¹.

adsorption in mass transfer to the electrode surface, necessary for current flow).

Electrochemical Determination of GSH

Since amperometry under stirred conditions has a higher sensitivity than other electrochemical techniques, it was used to GSH quantitation at rotating CuCoHCF/GO /GCE. Hydrodynamic amperograms were recorded during successive additions of GSH, while the potential of modified electrode was kept at 0.9 V vs. Ag/AgCl (Fig. 7A). The electrode response was linear within the concentration range of 3.3×10^{-7} - 5.3×10^{-6} M and 5.3×10^{-6} - 5.5×10^{-5} M (Fig. 7B). The limit of detection (LOD) was calculated as 2.5×10^{-7} M (3S/N). The analytical characteristics of the proposed electrode were compared with some of the previously reported sensors for GSH (Table 1). As seen, the

linear range and LOD of the present work are comparable with many of the previous reports. Moreover, available and inexpensive materials were used for electrode modification. The robustness and stability of the electrode are partly due to the presence of CuCoHCF which is quite stable in pH 3, and on the presence of GO as the substrate.

Stability and Reproducibility

One of the advantages of CuCoHCF/GO /GCE for amperometric detection of GSH was its high stability (Fig. 8) during a long period (> 6 min.). The inorganic complexes of MHCs have the advantage of robustness and stability in the field of electrode modification. The repeatability was evaluated for 5 successive measurements of a GSH solution (0.48 mM) by the proposed method as relative standard deviation (RSD%), which was 3.02%. When three different

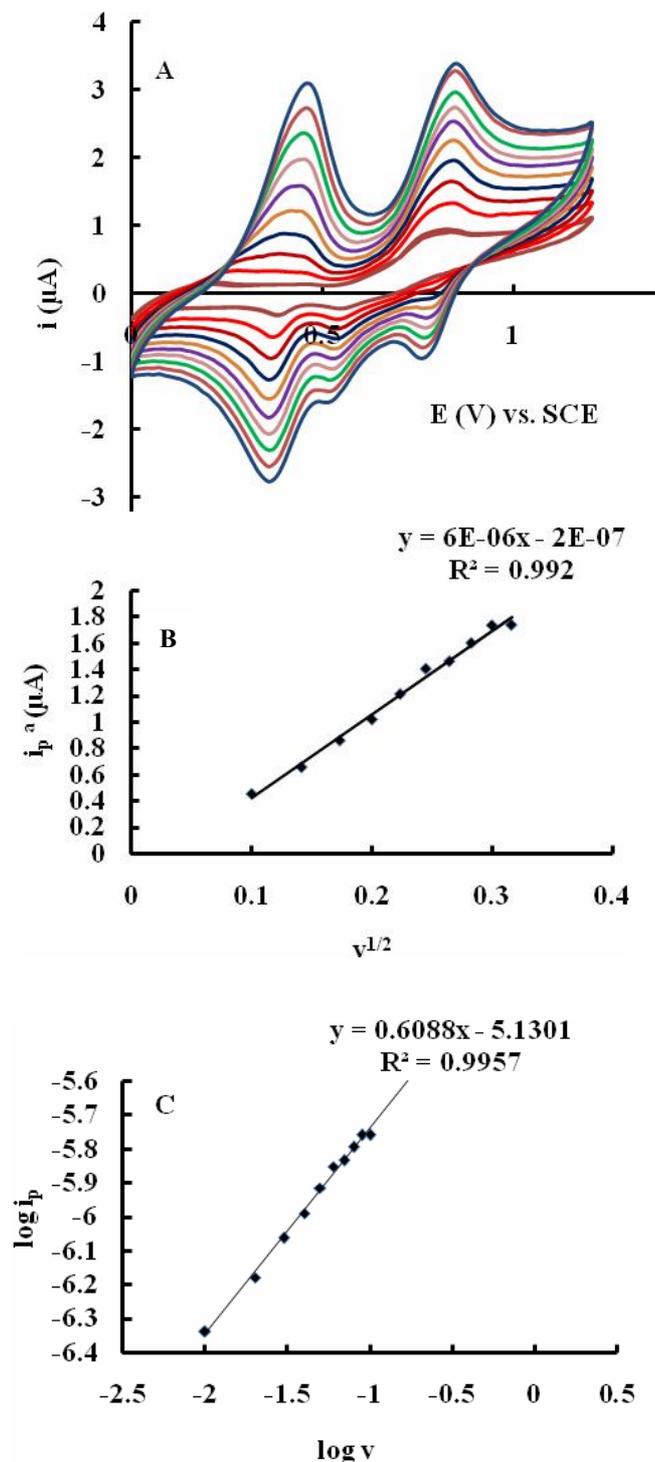


Fig. 6. (A) Cyclic voltammetry on GO/CuCoHCF/GCE at different scan rates (0.01-0.1 V s⁻¹) in the presence of GSH (0.48 mM) in PBS (0.1 M pH 3); (B) plot of anodic peak current against scan rate; (C) plot of $\log(i_p^a)$ against $\log(v)$.

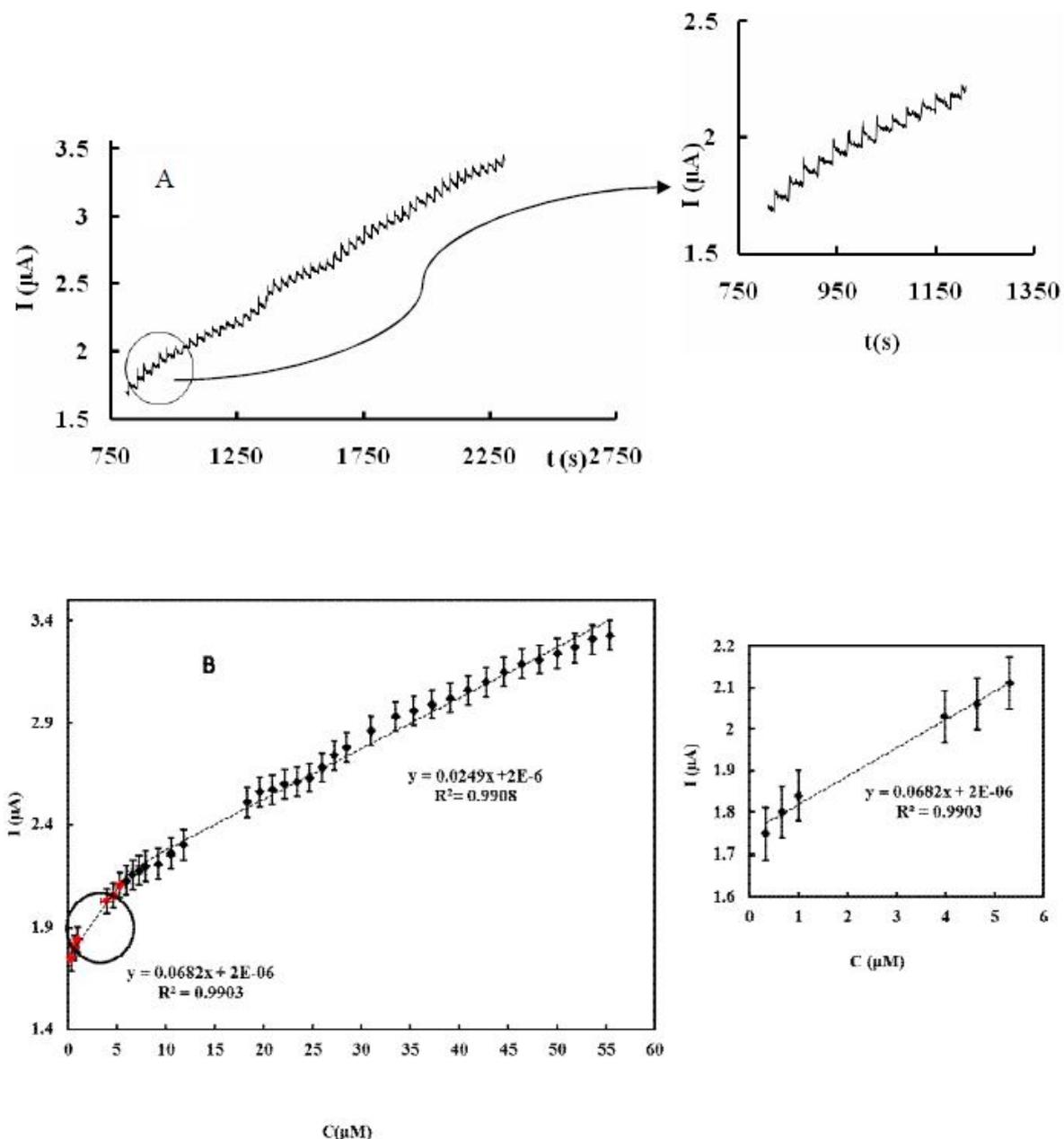


Fig 7. (A) Amperometric response of GO/CuCoHCF/GCE to successive additions of GSH to PBS (0.1 M, pH 3). (B) Calibration curve. Applied potential 0.9 V (against Ag/AgCl, KCl M), rotating rate of 1500 rpm, and interval time 0.1 s.

electrodes were used, RSD% for GSH determination was 3.34%. These results indicate that GO/CuCoHCF/GCE has a good stability and reproducibility.

Study of Interferences

Under the optimum conditions, the influence of various potentially interfering compounds was studied on the

Table 1. Comparison of CuCoHCF/GO/GCE with some Recently Reported Sensors for GSH Analysis

Electrode	Modifier (s)	Method	pH	Detection limit (μM)	Linear range (μM)	Ref. No.
Carbon paste	2,7-BFEF ^a	DPV	7.0	0.5	0.92-11	[58]
Carbon paste	Ferrocene	DPV	7.0	2.1	2.2-3000	[59]
Carbon paste	MWCNT ^b /Chlorpromazine	DPV	4.0	0.16	0.3-18.3	[60]
Glassy Carbon EPPGE ^c	Carbon nanotubes -	Amperometry CV	7.0 7.0	0.2 2.7	0.4-16.4 10-80	[61] [62]
Carbon paste	MWCNT/p-Aminophenol	SWV	5.0	0.09	0.2-100	[63]
Glassy Carbon	Nickel(II) oxide nanoparticles	DPV	7.2	-	200-6000	[64]
Carbon paste	ZnO/CNTs/BCB ^d	SWV	7.0	0.002	0.006-161	[17]
Carbon paste	DHB ^e ,CNT	DPV	7.0	0.07	0.4-25.0 25.0-700.0	[16]
Glassy Carbon	CuCoHCF/GO	Amperometry	3.0	0.25	0.3-5 5-55	Present work

^a2,7-Bis(ferrocenyl ethyl)fluoren-9-one. ^bMultiwalled carbon nanotubes. ^cEdge plane pyrolytic-graphite electrode. ^dZnO/carbon nanotubes/4,5-bis(4-chloroanilino)-1,2-benzendiol. ^e(E)-3-((2-(2,4-dinitrophenyl)hydrazono)methyl)benzene-1,2-diol.

determination of GSH. The tolerance limit was defined as the maximum concentration of the interfering substance that caused an error less than 5% for the determination of GSH. The current signal for GSH was not significantly affected in the presence of asparagine, arginine, histidine, glycine, glucose, urea and NaCl (Fig. 9). Although ascorbic acid

and cysteine show interference, they are not present at significant levels in hemolysed erythrocyte samples. In human blood, more than 99.5% of GSH is localized in erythrocyte, while 97% of cysteine is present in plasma. Furthermore, the GSH content is higher than 90% of total thiol-containing compounds in blood, therefore thiol

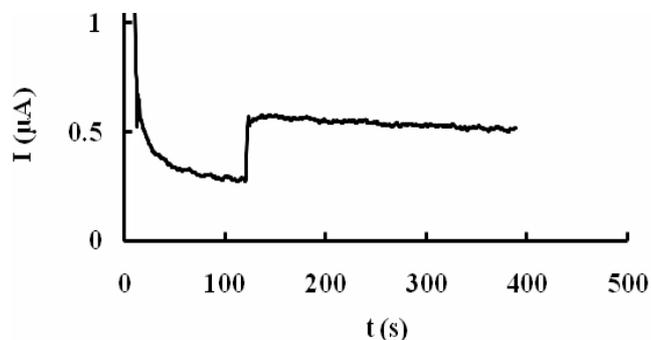


Fig. 8. Stability of current response to GSH (3.32 μM) at CuCoHCF/GCE. Applied potential 0.9 V (against Ag/AgCl, KCl M), rotating rate of 1500 rpm, and interval time 0.1 s.

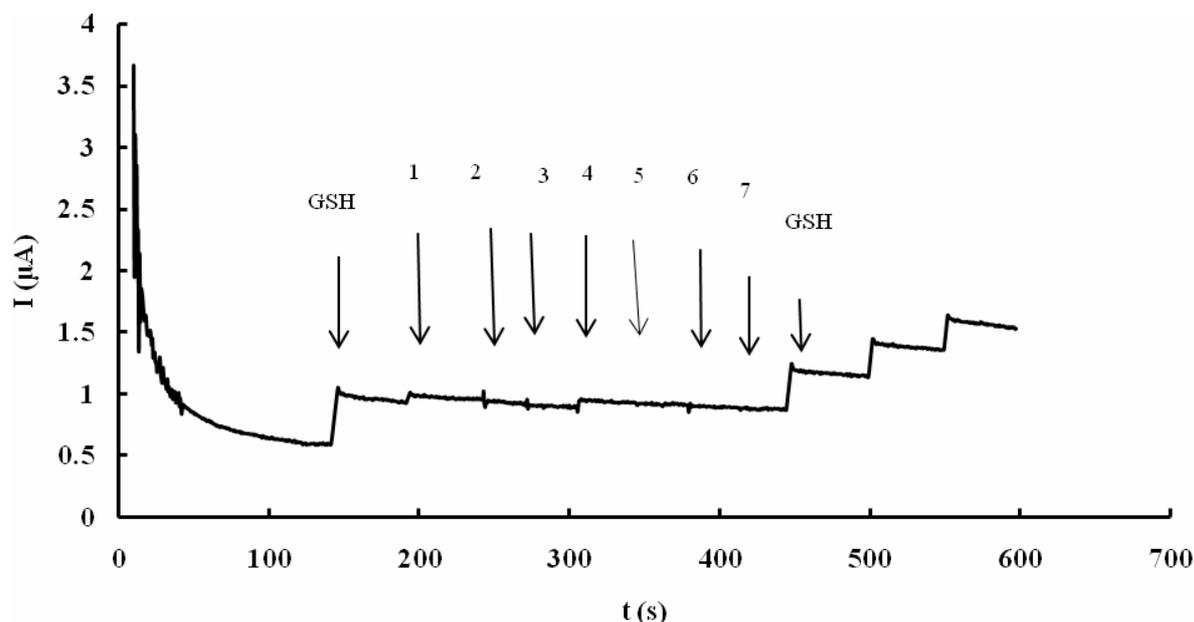


Fig. 9. Influence of various substances as potential interfering compounds on the determination of GSH (3.3 μM) at pH 3.0. (1) Asparagine, (2) Arginine, (3) Hystidine, (4) Glycine, (5) Glucose, (6) NaCl and (7) Urea. Applied potential 0.9 V (against Ag/AgCl, KCl M), rotating rate of 1500 rpm, and interval time 0.1 s.

compounds in whole blood can be regarded as GSH [54].

Determination of GSH in Human Erythrocyte Samples

The GSH content of human erythrocytes was estimated

by the proposed method and was compared with standard spectrophotometric method [52] for GSH determination. Successive amounts of standard solution of GSH were added to the sample and the current was measured in a hydrodynamic amperometry regime (Fig. 10A). From the

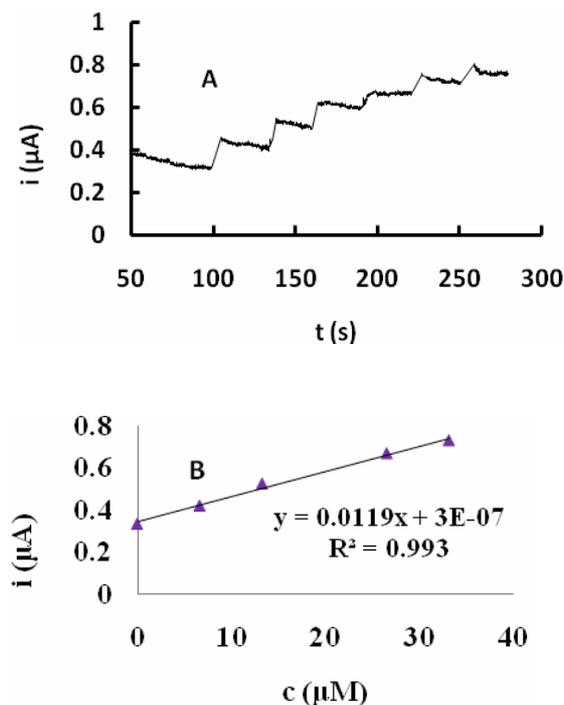


Fig. 10. (A) Amperometric response of CuCoHCF/GO/GCE for human erythrocytes sample. Applied potential 0.9 V (against Ag/AgCl, KCl M), rotating rate of 1500 rpm, and interval time 0.1 s. (B) Standard addition plot.

standard addition plot (Fig. 10B) the concentration of GSH in erythrocyte sample was calculated as $57.6 \pm 6.84 \mu\text{M}$ (three replicate measurements). Comparison with the results of the standard spectrophotometric method ($59.6 \pm 1.08 \mu\text{M}$) showed a good recovery of 96.64%.

CONCLUSIONS

In the present work, a glassy carbon electrode was modified with graphene oxide (GO) nanosheets and a hybrid of copper-cobalt hexacyanoferrate (CuCoHCF). The prepared electrode showed electrocatalytic properties against the oxidation of glutathione. The calibration curve was obtained by amperometry in a linear range of 3.33×10^{-7} - 5.3×10^{-6} and 5.96×10^{-6} - 5.54×10^{-5} M of glutathione with a limit of detection of 2.5×10^{-7} M. The current response toward glutathione was quite stable and reproducible, so, it was applied for the analysis of glutathione in hemolysed erythrocyte sample, successfully.

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