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Electrocatalytic Determination of Glutathione Using Multiwall Carbon Nanotubes Paste Electrode as a Sensor and Isoprenaline as a Mediator

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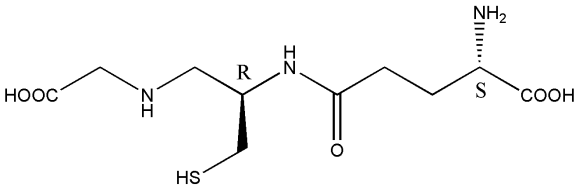
In this study, we propose isoprenaline (ISPT) as a new mediator for the rapid, sensitive, and highly selective voltammetric determination of glutathione (GSH) using multiwall carbon nanotubes paste electrode (MWCNTPE). It has been shown by direct current cyclic voltammetry, double step chronoamperometry and electrochemical impedance spectroscopy (EIS) that this modified electrode can catalyze the oxidation of GSH in aqueous solution. The kinetic parameters of the system including electron transfer coefficient, and catalytic rate constant were also determined using the electrochemical approaches. In addition, linear sweep voltammetry (LSV) was used for quantitative analysis. LSV showed wide linear dynamic range (0.5 – 300.0 μM GSH) with a detection limit of 0.09 μM GSH. Finally, this method was also examined as a selective, simple and precise electrochemical sensor for the determination of GSH in real samples such as hemolysed erythrocyte, tablet and urine.

Keywords: Glutathione determination, Isoprenaline, Hemolysed erythrocyte, Multiwall carbon nanotubes paste electrode, Sensor

1. INTRODUCTION

Glutathione (scheme 1) is the major intracellular thiol found in animals [1]. It is a tripeptide known to be involved in many biological processes [2]. Glutathione also has been used as a component of complex cosmetic preparations; in particular, its applications as antioxidant in the skin lipids protection [3] and for selective suppression of skin hyperipigmentation have been reported [4]. So,

determination of GSH in single human erythrocytes can potentially benefit clinical diagnosis at the early stages of disease.



Scheme 1. The chemical structures glutathione.

A number of methods have been proposed for the determination of GSH that include titrimetry [5], spectrophotometry [6,7], spectrofluorimetry [8-10] high performance liquid chromatography (HPLC) [11, 12], capillary zone electrophoresis [13], proton nuclear magnetic resonance (^{1}H NMR) [14], enzymatic method [15], flow injection analysis [16], and electrochemical methods [17-20]. Electrochemical methods have shown remarkable advantages in the analysis of different compounds in real samples. These advantages are mainly due to the simplicity, low cost and relatively short analysis times of these compounds as compared to chromatography [21-41].

Nanotechnology has become one of the most interesting disciplines in science and technology today [42-62]. The intense interest in nanotechnology is being driven by various interesting fields and is leading to a new industrial revolution. Carbon nanotubes (CNT) are an important nano structural that used building blocks of nanotechnology. With one hundred times the tensile strength of steel, thermal conductivity better than all but the purest diamond, and electrical conductivity similar to copper, but with the ability to carry much higher currents, they seem to be a very interesting material [63-85]. Since their discovery in 1991 [86], CNTs have generated great interest for future applications based on their field emission and electronic transport properties [87], their high mechanical strength [88] and high conductivity [89, 90]. The modification of electrode substrates with CNTs for use in analytical sensing has been documented to result in low detection limits, high sensitivities, reduction of over-potentials, and resistance to surface fouling [91-96].

In this study, we proposed ISPT as a mediator for the rapid, sensitive, and highly selective voltammetric determination of GSH on the surface of a multiwall carbon nanotubes paste electrode. The results showed that the catalytic current depends on the concentration of GSH. Cyclic voltammetry (CV), electrochemical impedance spectroscopy and double potential step chronoamperometry are employed to establish the electrocatalytic behavior of ISPT. The proposed method is selective, sensitive, and fast for the determination of GSH in real samples such as tablet, urine and hemolyzed erythrocyte.

2. EXPERIMENTAL

2.1. Apparatus and reagents

All the voltammetric measurements were performed using an Autolab PGSTAT 302N, potentiostat/galvanostat (Utrecht, The Netherlands) connected to a three-electrode cell, Metrohm

(Herisau, Switzerland) Model 663 VA stand, linked with a computer (Pentium IV, 1,200 MHz) and with Autolab software. A platinum wire was used as the auxiliary electrode. MWCNTPE and $Ag/AgCl/KCl_{sat}$ were used as the working and reference electrodes, respectively. The electrode prepared with carbon nanotubes was characterized by scanning electron microscopy (SEM) (Seron Tech. AIS 2100). A digital pH/mV-meter (Metrohm model 710) was applied for pH measurements. Spectrally pure graphite powder (particle size <50 μm) from Merck and multiwall carbon nanotubes (>90% MWCNTs basis, d × l = (110–70 nm) × (5–9 μm) from Fluka were used as the substrate for the preparation of the carbon paste electrode.

2.2. Preparation of the electrode

Graphite powder (0.900 g) was dissolved in diethyl ether and hand mixed with 0.100 g carbon nanotubes in a mortar and pestle. The solvent was evaporated by stirring. A syringe was used to add paraffin to the mixture, which was mixed well for 40 min until a uniformly wetted paste, was obtained. The paste was then packed into a glass tube. Electrical contact was made by pushing a copper wire down the glass tube into the back of the mixture. When necessary, a new surface was obtained by pushing an excess of the paste out of the tube and polishing it on a weighing paper.

2.3. Preparation of real samples

Human whole blood samples were obtained from the Isfahan University Health Center. Erythrocytes were separated from whole blood samples by removing the plasma. The sample thus obtained (2.0 mL) was first centrifuged for 10 min at 3000 rpm. The supernatant (plasma) was discarded and the rest was mixed with 5 mL of 0.9% NaCl solution. The solution was centrifuged for another 5 min at 3000 rpm and the supernatant (diluted plasma) was again discarded. The washing procedure with NaCl solution was repeated three times in order to remove almost all the plasma.

Erythrocyte pellets were hemolysed with water (1:1, v/v). For protein precipitation, the hemolysate was mixed with 5–sulfosalysilic acid (10%, m/v) at a ratio of 2:1 (v/v). The mixture obtained was centrifuged under the same conditions described above. Then, the supernatant was divided into two parts, one for spectrophotometric determination and the other for use with the proposed electrochemical method. For spectrophotometric measurement of the Ellman, a reference method [97] was used which is based on the reaction of glutathione with DTNB (Ellman's reagent), generating 2-nitro-5-mercapto-benzoic acid. Absorbance was monitored spectrophotometrically at 412 nm.

Urine samples were stored in a refrigerator immediately after collection. Ten milliliters of each sample was centrifuged for 15 min at 1500 rpm. The supernatant was filtered using a 0.45 μm filter and then diluted five times with universal buffer solution (pH 4.0). The solution was transferred into the voltammetric cell to be analyzed without any further pretreatment. Standard addition method was used for the determination of GSH in real samples.

Tablet solution was prepared by completely grinding and homogenizing five tablets of

glutathione, labeled 100 mg per tablet (Chongqing Yaoyou Pharmaceutical Co., Ltd.). Then, 10 mg of each tablet powder was accurately weighed and dissolved in 100 mL water by ultrasonication. After mixing completely, the mixture was filtered on an ordinary filter paper, 10 mL of which was subsequently transferred into a 100–mL volumetric flask and diluted to the mark with water. Then, 1.0 mL of the solution plus 4.5 mL of the buffer (pH 4.0) was used for analysis using the standard addition method.

2.4. Optimization of ISPT concentration

The influence of ISPT concentration on the peak currents was studied in the concentration range of 50-300 μM ISPT at pH 4.0. The results showed that by increasing the ISPT concentration up to 200 μM the net peak current increased, whereas further increasing the concentration of ISPT caused a decrease in the magnitude of the peak current. Therefore, 200 μM was selected as the optimal ISPT concentration.

3. RESULTS AND DISCUSSION

3.1. Characteristics of the MWCNTPE

Figure 1 shows SEM images for MWCNTPE and CPE. As can be seen at a surface of CPE (Fig. 1A), the layer of irregularly flakes of graphite powder was present and isolated with each other. After multiwall carbon nanotubes (MWCNTs) added to carbon paste, it can be seen that MWCNTs were distributed on the surface of electrode with special three-dimensional structure (Fig. 1B), indicating that the MWCNTs were successfully modified on the MWCNTPE.

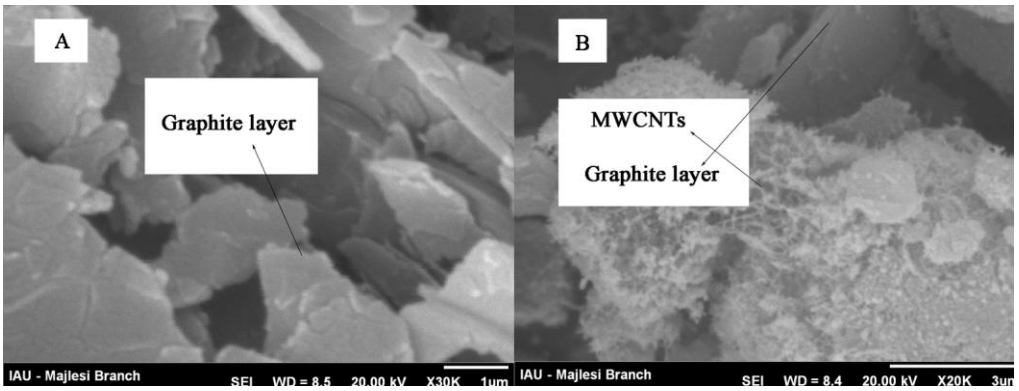


Figure 1. SEM image of A) CPE and B) MWCNTPE.

Figure 2 (insert) shows the cyclic voltammograms of ISPT at MWCNTPE in the universal buffer (pH 4.0) at a various scan rates.

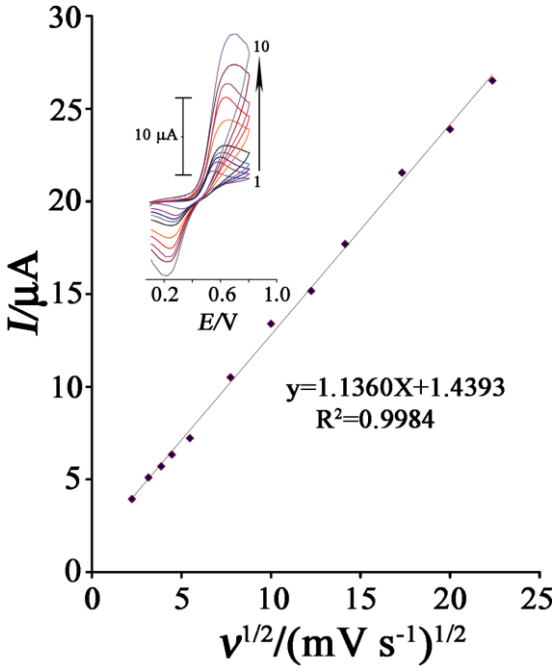


Figure 2. Plot of I_{pa} versus $v^{1/2}$ for the oxidation of ISPT at the surface of MWCNTPE. Inset) Cyclic voltammograms of 200 μM ISPT at various scan rates: (1) 5; (2) 10; (3) 15; (4) 20; (5) 30, (6) 60, (7) 100 , (8) 150, (9) 200 and (10) 300 mV s^{-1} in 0.04 M buffer solution (pH 4.0).

The experimental results showed well-defined and reproducible anodic and cathodic peaks related to $ISPT_{Red}/ISPT_{Ox}$ redox couple with quasi-reversible behavior, and with a peak separation potential of ΔE_p=180 mV (E_{pa}–E_{pc}). These cyclic voltammograms were used to examine the variation of peak current versus the sweep. The plots of anodic peak currents against the sweep rate show that the I_p values vary linearly with $v^{1/2}$ at all scan rates (Fig 2).

3.2. Electrocatalytic oxidation of GSH

The voltammetric behavior of the ISPT in the buffer solution (pH 4.0) is shown in Fig. 3a. The cyclic voltammetric responses for the electrochemical oxidation of 200 μM of GSH at MWCNTPE (curve c), and at the carbon paste electrode (curve b), in the presence of mediator, curves d and e are as c, b respectively, without ISPT. As can be seen, the anodic peak potentials for the oxidation of GSH at MWCNTPE in the presence of mediator (curve c) is about 629, whereas this potential is at 639 mV when using carbon paste electrode with ISPT (curve b). On the other hand, the oxidation GSH does not takes place at the surface of a carbon nanotubes paste electrode and carbon paste electrode without ISPT up to +1.0 V. Similarly, when we compared the oxidation of GSH at the surface of MWCNTPE (curve c) and carbon paste electrode with mediator (curve b), it was observed that a dramatic enhancement of the anodic peak current occurred at MWCNTPE *vs.* the value obtained with carbon paste electrode. In other words, the data obtained clearly show that the combination of MWCNTPE and the mediator (ISPT) definitely improve the characteristics of the electrode for the oxidation of

GSH. On the basis of the information, we suggest the electrocatalytic mechanism for the oxidation of GSH [98-110].

The influence of scan rate on the electrocatalytic oxidation of 200 μM GSH at MWCNTPE in the presence of ISPT was investigated by cyclic voltammetry. The oxidation peak potential shifted towards a more positive potential with increasing scan rate, confirming the kinetic limitation of the electrochemical reaction. In addition, a plot of peak height (I_p) against the square root of scan rate ($\nu^{1/2}$) in the range of 3–30 mV s^{-1} was constructed. The regression equation for this plot is: I_p = 2.6364X+2.9392 with R^2 = 0.9955. The linear dependence of I_p on the square root of scan rate ($\nu^{1/2}$) confirmed that at sufficiently high overpotentials, the process is diffusion rather than surface controlled [111-125].

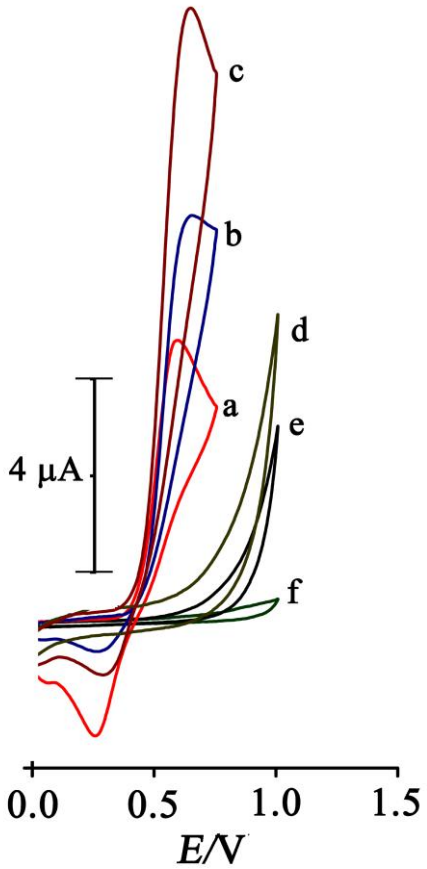


Figure 3. Cyclic voltammograms of 200 μM ISPT at the surface of MWCNTPE in 0.04 M universal buffer (pH 4.0) at a scan rate of 10 mV s^{-1} in the absence (a) and in the presence of 200 μM GSH (c). (c) as (b) for the carbon paste electrode. (d) as (c) and (e) as (b) for the unmodified electrode (and in the absence of ISPT). (f) For the buffer solution at the surface of unmodified electrode (carbon paste electrode).

In continuation of our studies, we used the Tafel plot to determine the electron transfer coefficient (α) in the catalytic oxidation process (Fig. 4). The slope of the Tafel plot was equal to n(1−

α)*F*/2.3*RT*, which came up to 9.1577 V decade^{-1}. We obtained the value of *n*α equal to 0.46. Assuming that *n* = 1, then α=0.46.

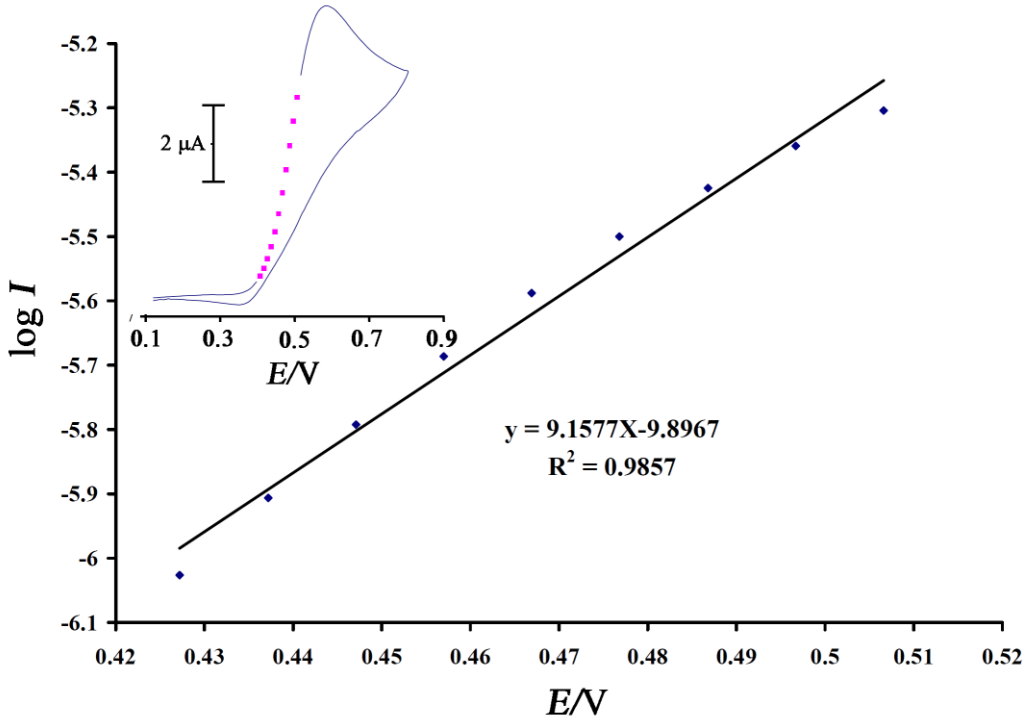


Figure 4. Tafel plot 200 μM ISPT at the surface of MWCNTPE in 0.04 M universal buffer (pH 4.0) at a scan rate of 3 mV s^{-1} in the presence of 200 μM GSH.

For determination of the diffusion coefficient and the catalytic reaction rate constant of GSH, double potential step chronoamperometry was used with MWCNTPE in the presence of mediator. Figure 5A shows the current–time curves of MWCNTPE in the presence of mediator by setting the electrode potential at 500 mV (first step) and 700 mV (second step) for different GSH concentrations. As can be seen, there is no net anodic current corresponding to the oxidation of the mediator in the presence of GSH. On the other hand, the forward and backward potential step chronoamperometry for the mediator in the absence of GSH shows symmetrical chronoamperogram with an equal charge consumed for the reduction and oxidation of the ISPT at the surface of MWCNTPE (Fig. 5D, a$^{/}$). On the other hand, the charge value associated with forward chronoamperometry in the presence of GSH is significantly greater than that observed for backward chronoamperometry (Fig. 5D, b$^{/}$–e$^{/}$). The linearity of the electrocatalytic current *vs.* $\upsilon^{1/2}$ shows that the current is controlled by GSH diffusion from the bulk solution toward the surface of the electrode, leading to a near Cottrellian behavior. A plot of I *vs.* $t^{-1/2}$ for different concentrations of GSH at the surface of MWCNTPE in the presence of ISPT yields straight lines (Fig. 5B) with different slopes which can be used to estimate the diffusion coefficient of GSH (D) in the ranges of 250 to 400 μM. The mean value of D for GSH was found to be 2.14×10^{-4} cm^2 s^{-1}.

The rate constant for the chemical reaction between GSH and ISPT at a surface of MWCNTPE, k_h, can be evaluated by chronoamperometry according to the method of Galus [126]:

$$I_C/I_L = \pi^{1/2}\gamma^{1/2} = \pi^{1/2}(kC_bt)^{1/2} \qquad (1)$$

where I_C is the catalytic current of GSH at MWCNTPE in the presence of ISPT, I_L is the limited current in the absence of GSH, and t is the time elapsed (s). The above equation can be used to calculate the rate constant of the catalytic process k_h. Based on the slope of the I_C/I_L *vs.* $t^{1/2}$ plots (Fig. 5C), k_h can be obtained for a given GSH concentration. Based on the values of the slopes, the average value of k_h was found to be equal to 5.36×10^2 mol L^{-1} s^{-1}. The value of k_h explains the sharp feature of the catalytic peak observed for catalytic oxidation of GSH at the surface of MWCNTPE in the presence of ISPT.

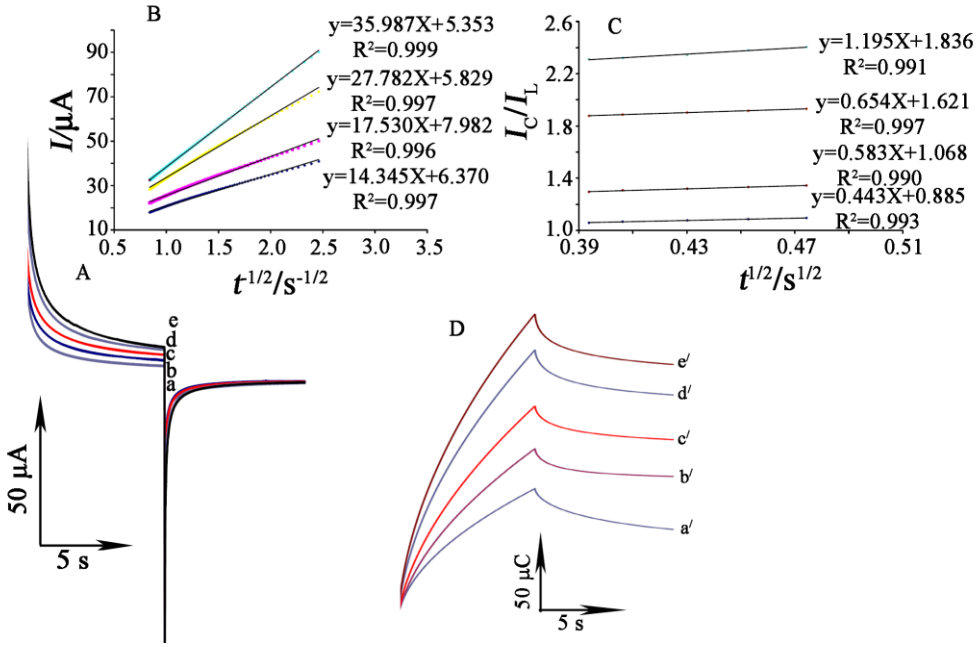


Figure 5. A) Chronoamperograms obtained at the MMWCNTPE in the absence a) and in the presence of b) 250, c) 300, d) 350 and e) 400 μM GSH in a buffer solution (pH 4.0). B) cottrell's plot for the data from the chronoamperograms. C) Dependence of I_c/I_L on the $t^{1/2}$ derived from the chronoamperogram data. D) The charge-time curves a') for curve (a); b') for curve (b); c′) for curve (c); d′) for curve (d) and (e′) for curve (e).

3.3. Electrochemical impedance spectroscopy studies

Electrochemical impedance spectroscopy as powerful techniques was also employed to investigate the oxidation of GSH [127, 128]. Figure 6 represents Nyquist diagrams of the imaginary impedance (Z_{im}) versus the real impedance (Z_{re}) of the EIS obtained at the modified electrode recorded at 0.52 V dc-offset in the absence (curve a) and the presence of 200 μM GSH (curve b) in 0.04 mol L^{-1}

universal buffer solution with pH 4.0. In the absence of GSH, the Nyquist diagram comprises a depressed semicircle at the high frequencies which can be related to the combination of charge transfer resistance of ISPT electrooxidation and the double-layer capacitance, followed by a straight line with the slope of near 45°. The latter is due to the occurrence of mass transport process via diffusion .

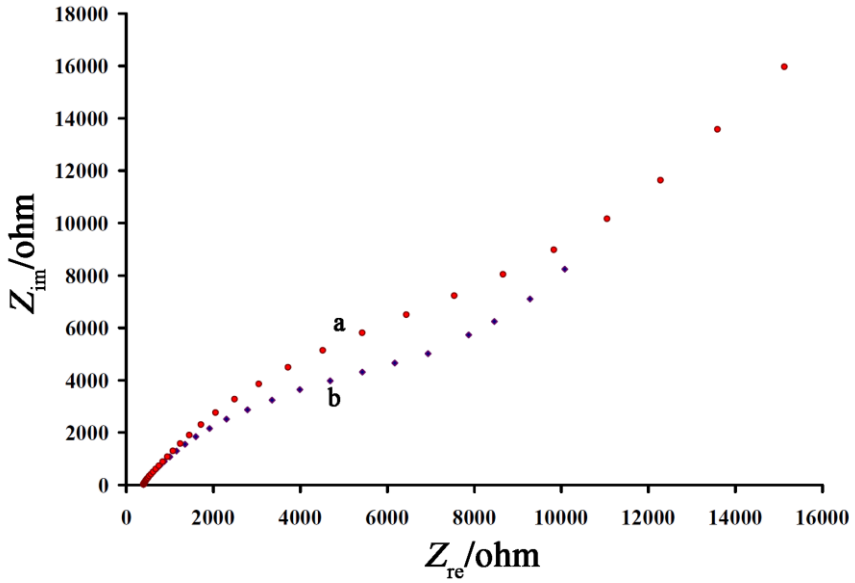
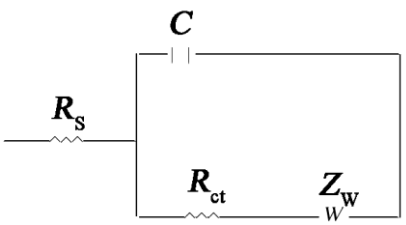


Figure 6 Nyquist diagrams of 200 µM ISPT on the MWCNTPE in the absence (a) and presence of (b) 200 µM GSH and in pH 4.0. Bias is 0.520 V with E_{ac} = 5 mV and frequency range of 10 *kHz* to 1 *Hz*.

The equivalent circuit compatible with the Nyquist diagram recorded in the absence and presence of GSH is depicted in scheme 2. In this circuit, R_s, C and R_{ct} represent solution resistance, a capacitance for the double-layer. W is a finite-length Warburg short-circuit term coupled to R_{ct}, which accounts for the Nernstian diffusion. In the presence of GSH, the diameter of the semicircle is decreased, confirming the electrocatalytic ability of the mentioned electrocatalyst for oxidation of GSH. This is due to the instant chemical reaction of GSH with the $ISPT_{(ox)}$ species.



Scheme 2. The equivalent circuit compatible with the Nyquist diagram recorded in Fig. 6

The catalytic reaction of GSH oxidation causes an increase in the surface concentration of $ISPT_{(Red)}$, and the charge transfer resistance becomes low, depending on the concentration of ISPT in

the solution. This behavior is consistent with the result of cyclic voltammetry and chronoamperometry (Figures 3 and 5). Impedance *W* elements can be expressed as [129]:

$$Z_W = Y_0^{-1}(j\omega)^{-1/2} \qquad (2)$$

Where Y_0 (the admittance parameter, S cm^{-2} s^{-n}) is parameter independent of frequency; $j = (-1)^{1/2}$ and □□= angular frequency = $2\pi f$.

4. INTERFERENCE STUDY

In order to evaluate the selectivity of the proposed method in the determination of GSH, the influence of various foreign species on the determination of 5.0 μmol L^{-1} GSH was investigated. The tolerance limit was taken as the maximum concentration of foreign substances which caused no more than ±5% relative error in the determination. The results are presented in Table 1. Although ascorbic acid shows interference, its interference can be minimized, if necessary, by using the ascorbic oxidase enzyme, which exhibits high selectivity for the oxidation of ascorbic acid.

Table 1. Interference study for the determination of 5.0 μmol L^{-1} GSH under the optimized conditions.

Species	Tolerance limits (W/W)
Glucose , Fructose, Lactose , Sucrose	1000
Li+, Cl-, Folic acid, NO3- , Hystidine, Alanine, Phenyl alanine, Methionine, Glycine, Methanol, Ethanol, Urea, SCN-,SO42-, Br-	800
Starch	Saturation
Ascorbic acid	5

5. DYNAMIC RANGE AND LIMIT OF DETECTION

Linear sweep voltammetry was used to determine the concentration of GSH. The results showed two linear segments with different slopes for GSH concentration: for 0.5–10.0 μmol L^{-1} of GSH, the regression equation was $I_p(\mu A) = (0.099\pm0.001)C_{GSH} + (6.845\pm0.915)$ (r^2=0.990, n=5), and for 10.0-300.0 μM GSH, the regression equation was $Ip(\mu A) = (0.0190\pm0.001)\ C_{GSH} + (8.897\pm0.957)$ (R^2 = 0.992, n = 6), where C_{GSH} is μM concentration of GSH. The detection limits, according to the definition of $Y_{LOD} = Y_B + 3\sigma$, was determined as 0.09 μM for GSH.

6. DETERMINATION OF GSH IN REAL SAMPLES

In order to evaluate the applicability of the modified electrode for measuring GSH in real samples, GSH values in human erythrocyte, tablet, and urine samples were determined using the

proposed method. In addition, the results were compared with those obtained from the spectrophotometric method [97] which is usually used as the standard method for glutathione determination. The results are reported in Table 2.

Table 2. Concentration values obtained from the proposed and Elman methods for GSH analysis in hemolysed erythrocyte, tablet and urine samples.

Sample	Proposed method (mmol L–1)	Elman method (mmol L–1)	Fex	Ftab, (0.05);2,2	tex	ttab (98%)
1. Hemolysed erythrocyte	5.32 ± 0.03	5.45 ± 0.09	6.2	19	2.3	3.8
2	4.25 ± 0.02	4.31 ± 0.06	5.5	19	2.0	3.8
3	5.55 ± 0.04	5.41 ± 0.07	6.0	19	2.1	3.8
4	4.45 ± 0.05	4.68 ± 0.08	7.0	19	2.7	3.8
5 Urine	Less than limit of detection	Less than limit of detection	–	–	–	–
6	0.04 ± 0.003	0.04 ± 0.008	4.9	19	1.9	3.8
7	0.07 ± 0.044	0.07 ± 0.084	–	–	–	–
Tablet	0.15 ± 0.021	0.14 ± 0.041	7.8	19	3.1	3.8
	0.20 ± 0.031	0.20 ± 0.063	–	–	–	–

F_{ex} Calculated F-value; Reported F value from F-test table with 95% confidence level and 2/2 degree of freedom; t_{ex} Calculated t; t_{tab} (98%) Reported t value from t-student test table with 98% confidence level.

7. CONCLUSION

A new votammetric sensor developed for the determination of GSH is very rapid, reproducible, highly selective and sensitive, and can be used for real sample analysis. This mediator shows excellent catalytic effects on the oxidation of GSH. It has been found that, with cyclic voltammetry, the oxidation of glutathione occurred at a potential about 629 mV on the surface of the multi wall carbon nanotubes paste electrode in the present of ISPT, while the oxidation GSH does not takes place at the surface of a carbon nanotubes paste electrode without ISPT as a mediator up to +1.0 V. The proposed method was also used as a selective, simple, and precise new sensor for voltammetric determination of GSH in real samples such as hemolysed erythrocyte, tablet and urine.

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