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Biosensing Approach for Glutathione Detection Using Glutathione Reductase (GR) With Multi-Walled Carbon Nanotubes on Gold Electrode

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MWCNTs/GR modified electrode which contains multi-walled carbon nanotubes (MWCNTs) and glutathione reductase (GR) has been synthesized on gold electrode. The presence of MWCNTs enhances the surface coverage concentration (Γ) and stability. The measured contact angle of treatment MWCNTs indicates that the sample had more hydrophilic on modified electrode surface. The modified electrode also exhibits a promising enhanced electrocatalytic activity towards the reduction of glutathione. The electrochemical activity of MWCNTs/GR modified gold electrode has been examined using electrochemical impedance spectroscopy (EIS). The cyclic voltammetry (CVs) has been used for the measurement of electroanalytical properties of analytes by means of modified electrodes. The sensitivity values of MWCNTs/GR modified gold electrode are higher than the values which are obtained for only GR modified electrode. We have studied the surface morphology of the ITO modified electrode using scanning electron microscopy (SEM) and atomic force microscopy (AFM), which revealed that GR is coated on MWCNTs. Finally, amperometric response has been used for detection of analytes at MWCNTs/GR film modified gold electrode.

Keywords: Multiwall carbon nanotubes; glutathione reductase; modified electrodes; electrocatalysis; glutathione.

1. INTRODUCTION

Glutathione (GSH) and glutathione disulfide (GSSG) play an important role in the metabolism of living cells [1-6]. It has gained much attention in recent years due to its vital biological functions such as involvement in bioreductive reactions, enzyme activity maintenance, amino acid transport, protection against oxidative/nitrosative stress and detoxification of drugs [5-16]. Glutathione exists in nature in the oxidized (GSSG) and reduced (GSH) forms [17]. Reduced glutathione is the main

nonprotein thiol which is found at high concentrations in many living cells. Under oxidative stress, glutathione in reduced form (GSH), can be converted to the oxidized form, GSSG, and rapidly reverts back to GSH by the action of the enzyme glutathione reductase (GR) [18-20]. It is a key enzyme in the conversion of GSSG to GSH in both prokaryotes and eukaryotes and maintaining the reduced state of the cell [21]. Glutathione reductase(GR) is a flavoenzyme of known structure [22] that catalyzes the NADPH-dependent reduction of oxidized glutathione via a disulfide exchange reaction involving two active site cysteine residues [23-28]. It is a dimer in solution held together by noncovalent interactions. GR accepts electrons from reduced NAD(P)H into noncovalently bound FAD to give the $FADH_2$ (reduced) enzyme form. The electrons from $FADH_2$ are then transferred to an internal disulfide bond formed between Cys42 and Cys47 in the active site. The reduced cysteines then catalyze reduction of oxidized glutathione via a disulfide exchange reaction. Importantly, the NADPH binding site and the Cys42-Cys47 disulfide (glutathione binding site) are on opposite faces of the FAD cofactor and the enzyme structure. Thus, in principle, the glutathione binding site could be occupied by metals and the FAD could still be reduced by NADPH [29].

GSH is over expressed in many tumor tissues and altered levels of GSH in plasma have been implicated in a number of pathological conditions, including Alzheimer's, Parkinson's diseases, diabetes, macular degeneration, HIV disease, chronic renal failure, malignant disorders, alcoholism and cataract formation [30-35]. Therefore, detection of GSH is of more and more significant importance. GSH can now be detected by some methods such as high performance liquid chromatography (HPLC), spectrofluorimetry, spectrophotometry, electrochemistry and capillary electrophoresis combined with electrochemical detection [36-45]. Electroanalytical techniques have been shown to provide a highly sensitive and selective approach for the detection of numerous compounds [46], and a variety of electroanalytical methods have been developed for the detection of GSH because it is electroactive [47-51]. As mentioned previously, common carbon electrodes such as glassy carbon show no signal corresponding to GSSG oxidation, and the signal for GSH is observed at a relatively high overpotential of ~1.0 V vs SCE [52]. Recently, it was reported that multi-walled carbon nanotubes (MWCNTs) could offer special physical and chemical properties such as increasing the surface area, mass transport, controlled release and catalysis [53-54]. A study explains the use of a functionalized carbon nanotube modified electrode coupled with HPLC as a detector for the determination of GSH and GSSG [55].

In this work, we designed glutathione reductase (GR) based on MWCNTs modified electrode for highly sensitive detection of GSH. Due to its excellent resistance to surface fouling and its renewable surface, the electrode is suggested for direct analysis of GSH and GSSG in blood serum samples.

2. EXPERIMENTAL

2.1. Materials

Multi-walled carbon nanotubes (Aldrich) was used as received. Glutathione reductase (GR,

EC1.6.4.2 from wheat germ, 0.05 U/mg solid), Glutathione reduced (GSH), 5,5'-dithiobis(2-nitrobenzoic acid)(DTNB), nicotinamide adenine dinucleotide phosphate (NADPH) were obtained from Sigma. All other chemicals used were of analytical grade and used without further purification. 0.1 M Phosphate buffer solutions (PBS) was used as supporting electrolyte. Aqueous solutions were prepared using doubly distilled deionized water and then deaerated by purging with high purity nitrogen gas for about 20 min before performing electrochemical experiments. Also, a continuous flow of nitrogen over the aqueous solution was maintained during measurements.

2.2. Apparatus

Cyclic voltammetry (CVs) were performed in an analytical system model CHI-1205B potentiostat. A conventional three-electrode cell assembly consisting of an Ag/AgCl reference electrode and a Pt wire counter electrode were used for the electrochemical measurements. The working electrode (area = 0.03 cm^2) was either an unmodified gold or a gold modified with only glutathione reductase (GR), or MWCNTs/glutathione reductase (MWCNTs/GR) films. In these experiments, all the potentials have been reported versus the Ag/AgCl reference electrode. The amperometric response using CHI-750 potentiostat. Gold electrode (geometric area 0.12 cm^2) obtained from Bioanalytical Systems (BAS) served as a working electrode. The morphological characterizations of the films were examined by means of SEM (Hitachi S-3000H) and atomic force microscopy (AFM) (Being Nano-Instruments CSPM5000). Electrochemical impedance spectroscopy (EIS) measurements were performed using an IM6ex Zahner instrument (Kroanch, Germany). The water contact angles of the MWCNTs were measured by the micro syringe drop method, using CMA110 system. The contact angle reported here was an averaged value of at least five measurements. All the solutions were purged with high purity nitrogen gas for about 20 min before performing electrochemical experiments. Also, a continuous flow of nitrogen over the aqueous solution was maintained during measurements. All the experiments were carried out at room temperature (≈ 25°C).

2.3. Preparation of modified electrode

2.3.1. Preparation of MWCNTs

There was an important challenge in the preparation of MWCNTs. Because of its hydrophobic nature, it was difficult to disperse it in any aqueous solution to get a homogeneous mixture. Briefly, the hydrophobic nature of the MWCNTs was converted in to hydrophilic nature by following the previous studies [56-57]. This was done by weighing 10 mg of MWCNTs and 200 mg of potassium hydroxide in to a ruby mortar and grained together for 4 hr at room temperature. Then the reaction mixture was dissolved in 10 ml of double distilled deionized water and it was precipitated many times in to methanol for the removal of potassium hydroxide. Thus obtained MWCNTs in 10 ml water was ultrasonicated for 6 hr to get a uniform dispersion. This functionalization process of MWCNTs was done to get a hydrophilic nature for the homogeneous dispersion, in water. This process not only

converts MWCNTs to hydrophilic nature but this helps to breakdown larger bundles of MWCNTs in to smaller ones also[58].

2.3.2. Preparation of Gold Electrodes Modified with MWCNTs/GR

Prior to modification, gold electrode was polished with 0.05 μm alumina on Buehler felt pads and then ultrasonically cleaned for about a minute in water. Finally, the electrode was washed thoroughly with double distilled water and dried at room temperature. The cleaned gold electrode was coated with 2μL of MWCNTs and the solvent allowed evaporating at room temperature. Then the electrode was immersed in fresh phosphate solution containing 1 mg mL^{-1} GR. The modified electrode was rinsed with supporting electrolyte and used for further studies.

3. RESULTS AND DISCUSSIONS

3.1. Electrochemical characterizations of MWCNTs/GR film

The water contact angles of MWCNTs were measured at 25℃ by the micro syringe drop method, and the reported results were the mean values of five times. The contact angle, a measure of the surface wettability, was used to determine the hydrophobicity and hydrophilicity.

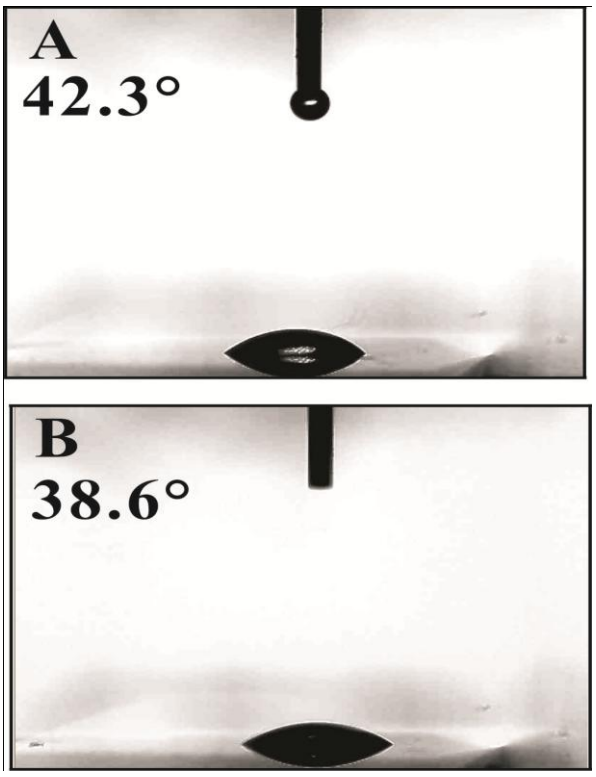

Figure 1. The water contact angles of MWCNTs were measured at 25℃ by the micro syringe drop method, shows (A) untreatment and (B) treatment MWCNTs dropped on ITO.

Fig 1. shows (A) untreatment and (B) treatment MWCNTs dropped on ITO. The measured contact angle of untreatment MWCNTs was close 42.3° and treatment MWCNTs shifted to 38.6°. This low contact angle indicates that the sample had more hydrophilic on surface.

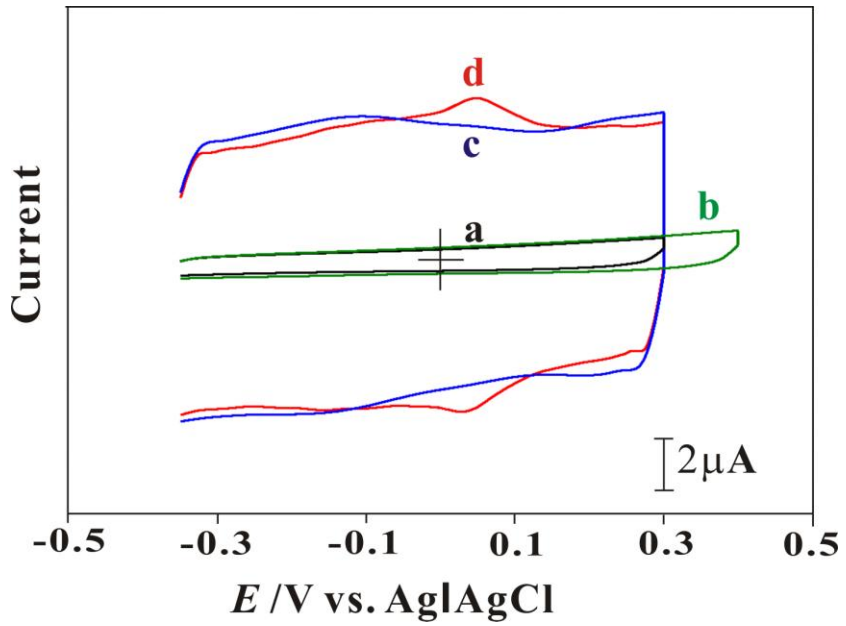

Figure 2. Cyclic voltammograms in 0.1M PBS (pH = 7.0) for different electrodes: (a) only GR; (b) bare gold electrode (c) MWCNTs and (d) MWCNTs/GR , Scan rate = 100mV s^{-1}.

Table. 1. Surface coverage concentrations (Γ) of GR at gold modified electrode.

Electrode type	Modified film	Γ (mol cm^{-2})
Au [a]	GR	9.62×10^{-12}
	MWCNTs	1.19×10^{-10}
	MWCNTs/GR	1.23×10^{-10}

A=0.03cm^2

[a] Studied using CV technique in PBS (pH 7.0).

In the following experiments, each newly prepared film on gold electrode has been washed carefully in deionized water to remove the loosely bounded GR on the modified gold electrode. It was then transferred to PBS for the other electrochemical characterizations. These optimized pH solutions have been chosen to maintain the higher stability (pH = 7.0). Fig. 2. shows different types (a) only GR, (b) bare gold electrode, (c) MWCNTs and (d) MWCNTs/GR. The corresponding cyclic voltammetric have been measured at 100 mVs^{-1} scan rate in the potential range of -0.35 to 0.3 V. The redox peak current at formal potential $E^{0'}$ = 0.06V represents the redox peak for GR (curve a). Curve (c) of figure shows cyclic voltammetric response of MWCNTs dispersion modified electrode. As can be seen, there is no obvious peak in the potential region studied. From this figure, comparison of curve (a) and curve

(d) , it is found that the presence of MWCNTs shows the catalytic effect on GR redox peak currents. Further, it has been observed that the presence of MWCNTs increases the overall back ground current, which is similar to that of previous studies [59-60]. These results are evident with the active surface coverage concentration (Γ) give in Table. 1 where, we can note the enhanced Γ of GR in the MWCNTs modified electrode. In this calculation, the charge involved in the reaction (Q) has been obtained from CVs and it has been applied in the equation $\Gamma = Q/nFA$. These values indicate that the presence of MWCNTs increased the surface area of the electrode, which in turn has increased the Γ of GR. The calculated values from the same table shows that, the overall percentage of increase in Γ of GR in MWCNTs film is 1.13×10^{-10} mol cm^{-2}. In these Γ calculations, the number of electrons involved in the GR redox reaction is assumed as two.

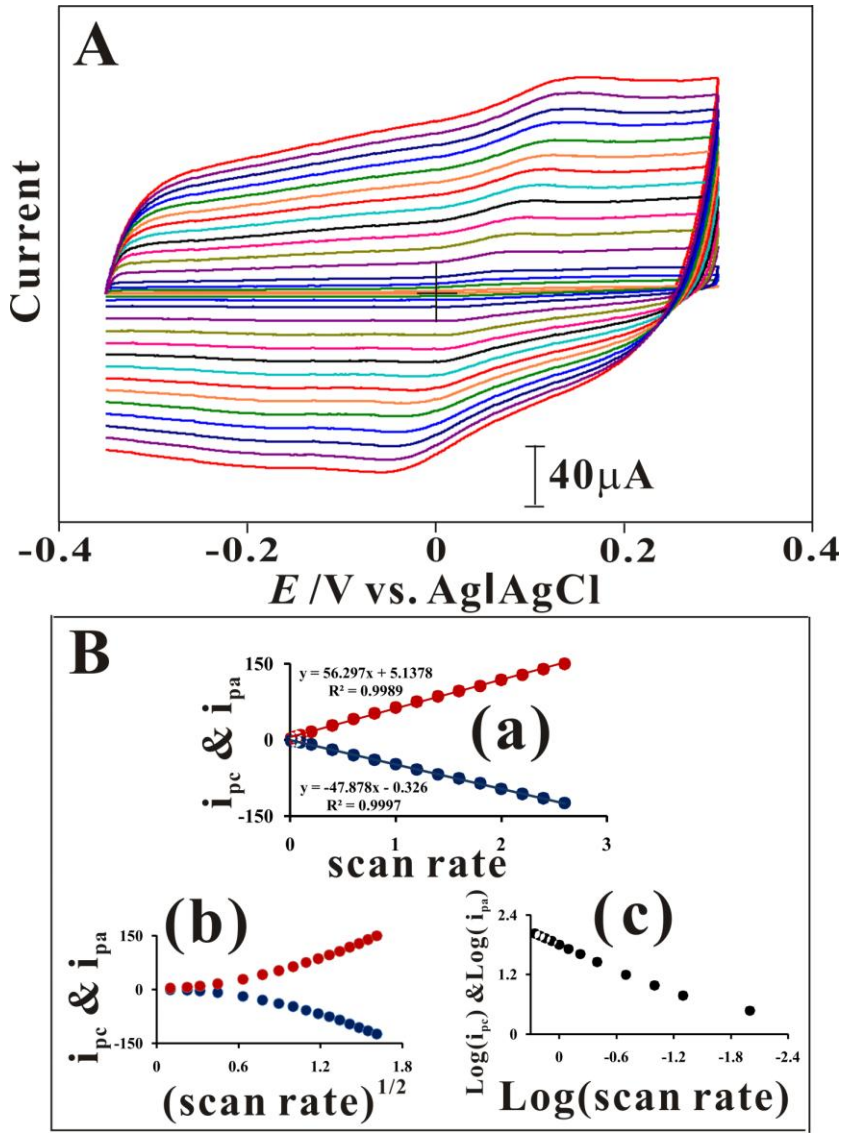

Figure 3. (A) Cyclic voltammograms of 0.1 M PBS (pH = 7.0) at MWCNTs/GR electrode at different scan rate from 10 mV s^{-1} to 2600 mV s^{-1}, respectively. Calibration curve for data in figure 2 (B) shows I_{pa} & I_{pc} vs. scan rate; I_{pa} & I_{pc} vs. scan rate$^{1/2}$; log (I_{pa}) & log (I_{pc}) vs. log (scan rate).

Immobilization of enzymes to solid electrode surface is a key step for the design, fabrication and performance of the biosensor, since it is well known that some enzymes retain their activity when they are immobilized [61]. Inorder to confirm stability of modified electrode, the cyclic voltammetric of the MWCNTs/GR electrode using PBS (ph = 7.0) at different scan rates (10 to 2600 mV/s) . Fig. 3 (A) have shown that the anodic and cathodic peak currents of both the film redox couples which have increased linearly with the increase of scan rates. Calibration curve for data in figure 3 (B) shows (a) I_{pa} & I_{pc} vs. scan rate, (b) I_{pa} & I_{pc} vs. scan rate$^{1/2}$ and (c) log(I_{pa}) & log(I_{pc}) vs. log(scan rate). The ratio of I_{pa}/I_{pc} from the inset has demonstrated that the redox process has not been controlled by diffusion. However, the ΔE_p of each scan rate reveals that the peak separation of composite redox couple increases as the scan rate is increased.

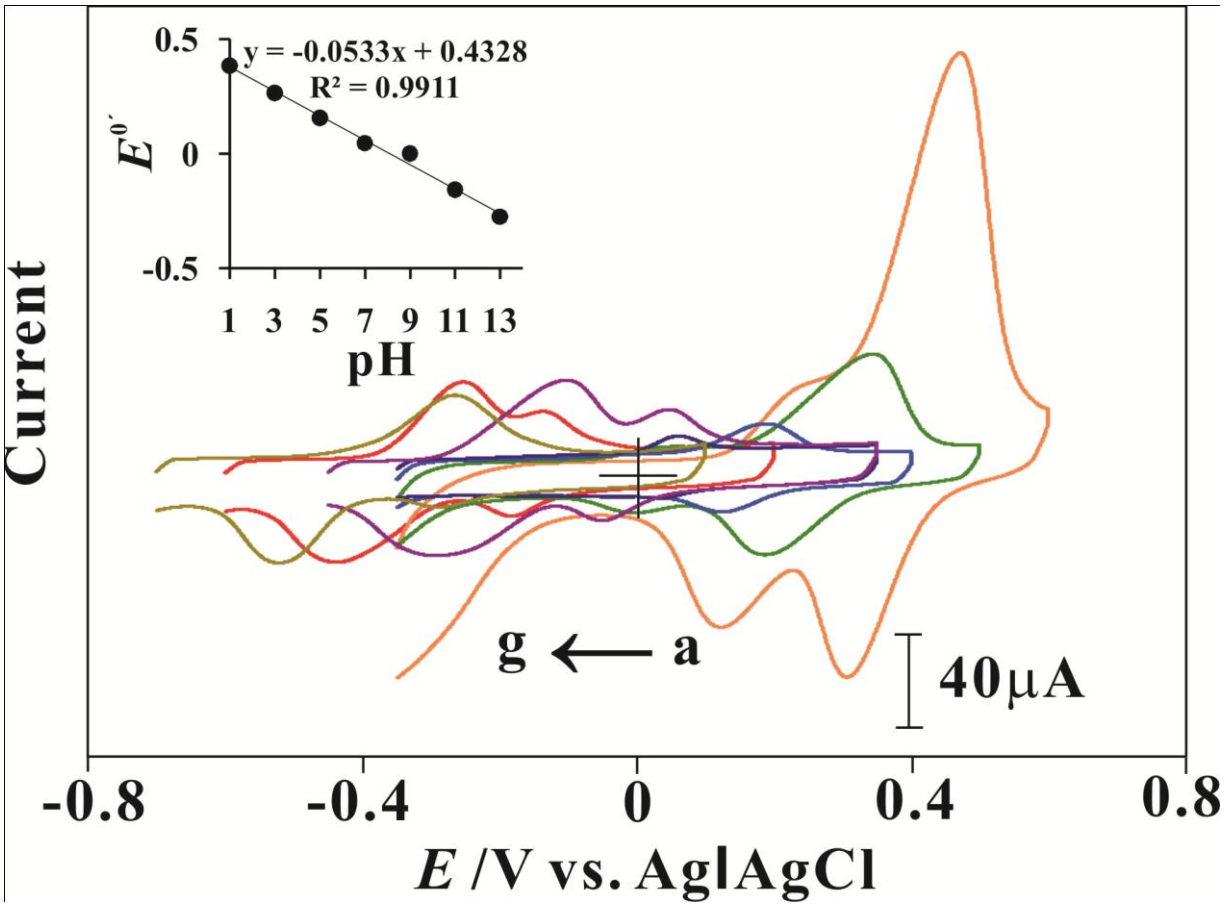

Figure 4. Cyclic voltammograms of the MWCNTs/GR transferred to various (a) 1, (b) 3, (c) 5, (e) 7, (d) 9, (f) 11, (g) 13 pH solutions. The inset shows the formal $E^{0'}$ vs. pH.

Fig. 4 shows the cyclic voltammetric of MWCNTs/GR on electrode obtained in PBS, then washed with deionized water and was transferred to various pH aqueous buffer solutions. This shows that the film is highly stable in the pH range between 1 to 13. The values of E_{pa} and E_{pc} depends on the pH value of the buffer solution. The inset in Fig. 4 shows the potential of MWCNTs/GR plotted over a pH range from 1 to 13. The response shows a slope of -53.3 mV/pH, which is close to that given by the Nernstian equation for equal number of electrons and protons transfer [62-63].

3.2. Morphological characterization of MWCNTs/GR film

Further, three different films; MWCNTs, only GR and MWCNTs/GR have been prepared on the inoxide electrode (ITO) electrode were characterized using SEM. From Fig. 5, it is significant that there are morphological differences between both the films.

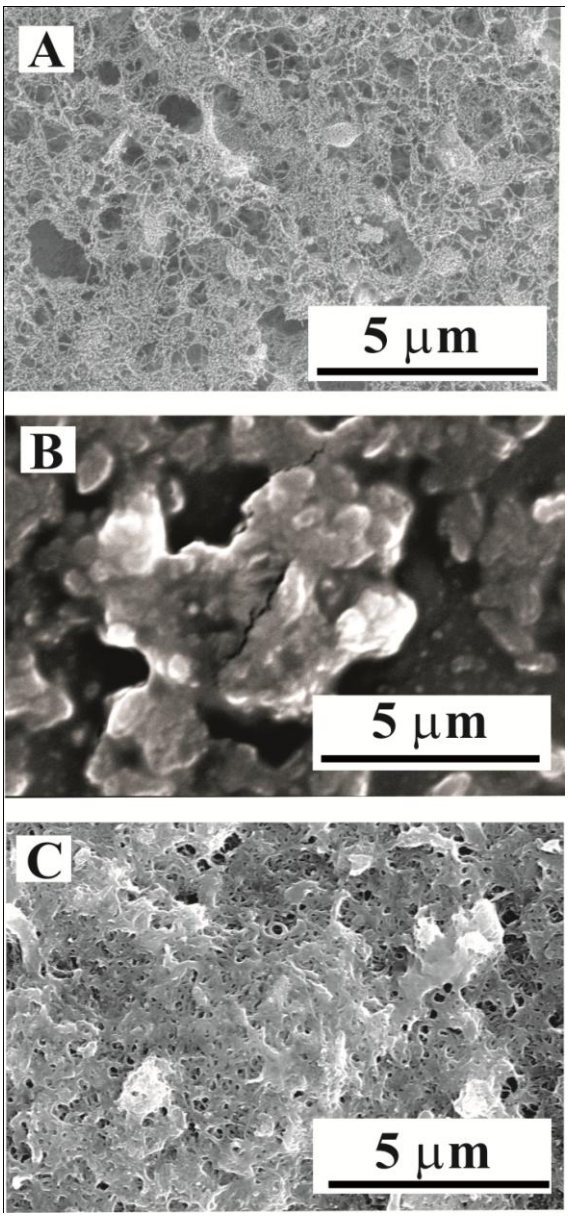

Figure 5. SEM images of (A) MWCNTs; (B) only GR and (C) MWCNTs/GR on ITO electrode.

It is a well known fact that the prolonged exposure to the electron beam will damage the GR films, so an at most care was taken to measure these images. The top views of nano structures Fig. 5 (A) on the ITO electrode surface shows MWCNTs on this electrode. Fig 5 (B) shows only GR. The MWCNTs/GR film in Fig. 5 (C) reveals that the GR had covered the entire MWCNTs to form MWCNTs/GR modified electrode. The same modified ITO electrodes have been used to measure the

AFM topography images of Fig. 6 (A) MWCNTs, (B) only GR and (C) MWCNTs/GR electrode. In all these cases the observed morphological structure is similar to that of SEM. We can clearly see that the immersed MWCNTs/GR have been gathered together and resemble like a circular one, respectively.

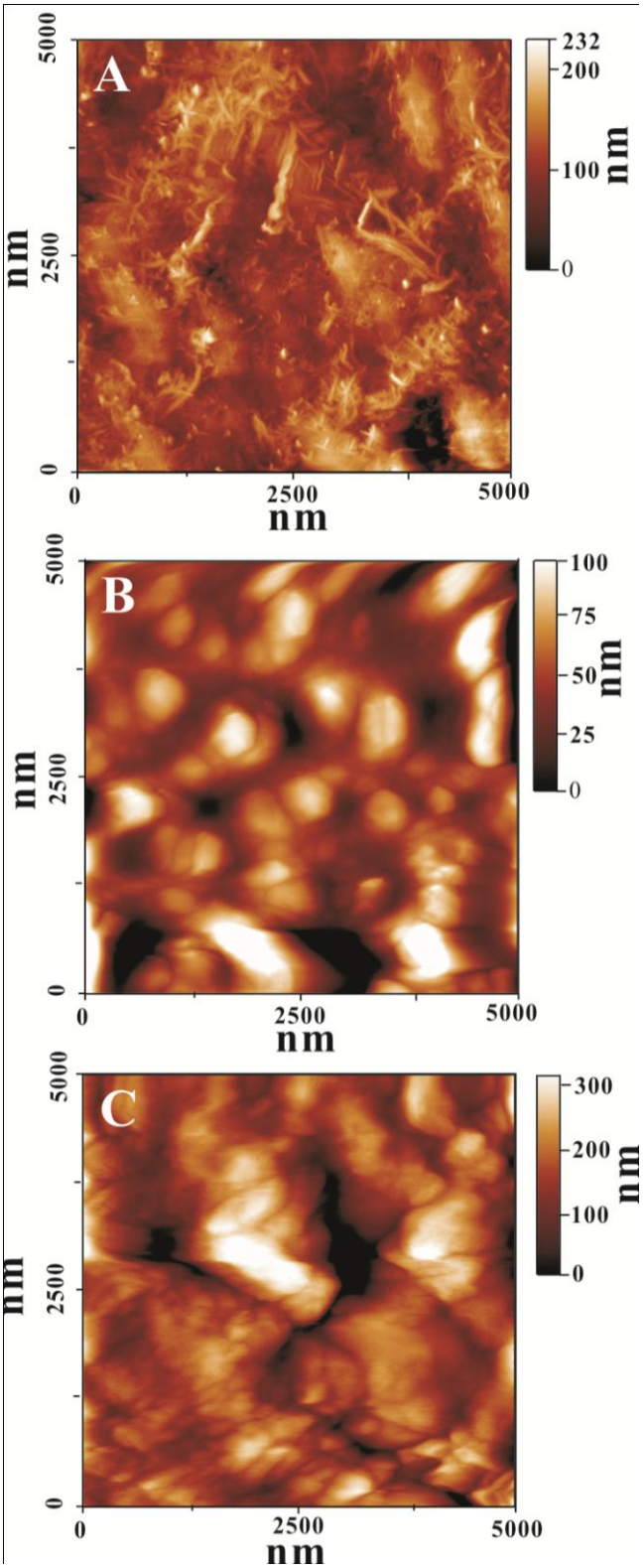

Figure 6. AFM images of (A) MWCNTs; (B) only GR and (C) MWCNTs/GR on ITO electrode.

3.3. Electrochemical impedance spectra (EIS) of Analysis

The electrochemical activity of MWCNTs/GR modified gold electrode has been examined using EIS technique. Impedance spectroscopy is an effective method to probe the features of surface modified electrodes. This study was employed to analyze detailed electrochemical activities of modified electrode with individual or mixed components.

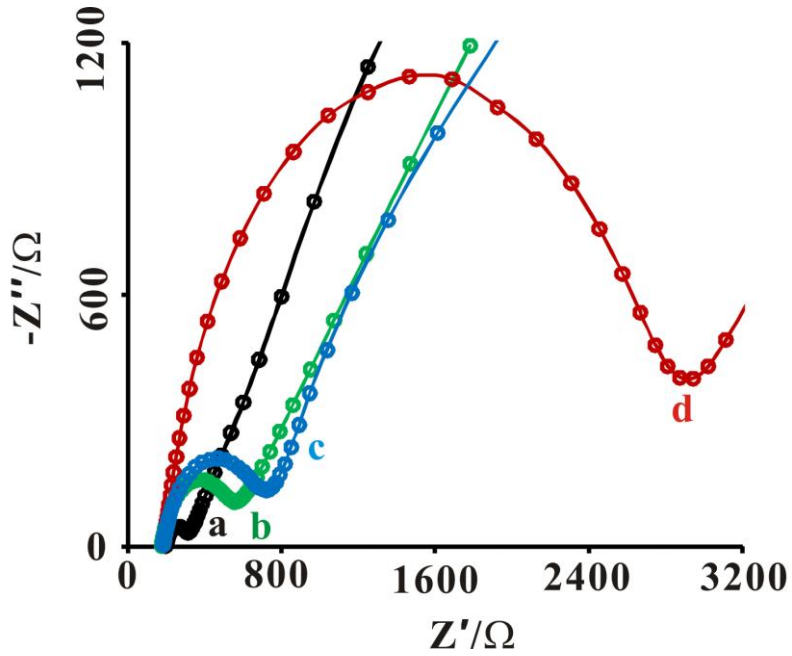

Figure 7. Electrochemical impedance spectra (EIS) of (a) MWCNTs, (b) only GR, (c) MWCNTs/GR and (d) bare gold electrode in pH 7.0 PBS containing 5×10^{-3} M $[Fe(CN)_6]^{-3/-4}$ (Amplitude: 5 mV).

The complex impedance can be presented as a sum of the real, Z′ (ω), and imaginary Z″(ω), components that originate mainly from the resistance and capacitance of the cell. From the shape of an impedance spectrum, the electron-transfer kinetics and diffusion characteristics can be extracted. The respective semicircle parameters correspond to the electron transfer resistance (R_{et}) and the double layer capacity (C_{dl}) nature of the modified electrode. Fig. 7. shows the Faradaic impedance spectra, presented as Nyquist plots (Z″vs. Z′) for the MWCNTs/GR, MWCNTs, GR modified gold electrode and bare gold electrode. The MWCNTs exhibits almost a straight line (a) with a very small depressed semicircle arc (R_{et}= 340 (Z′/Ω)) represents the characteristics of diffusion limited electron-transfer process on the electrode surface. At the same time, the GR modified gold electrode shows like a depressed semicircle arc with an interfacial resistance due to the electrostatic repulsion between the charged surface and probe molecule $Fe(CN)_6^{-3/-4}$ (b). This depressed semicircle arc (R_{et}=606 (Z′/Ω)) clearly indicates the lower electron transfer resistance behavior comparing with the bare gold electrode (d) (R_{et}=2940 (Z′/Ω)). MWCNTs/GR film modified gold electrode's R_{et} has been found as 756 (Z′/Ω). The increase in the value of electron transfer resistance (R_{et}) due to the coating of enzyme as a barrier on electrode surface. Thus, the electron transfer process will become as a slow process on the gold

electrode. Finally, these results clearly illustrate the electrochemical excellent activities of the MWCNTs/GR modified gold electrode, respectively.

3.4. Electrocatalytic of glutathione at MWCNTs/GR modified gold electrode

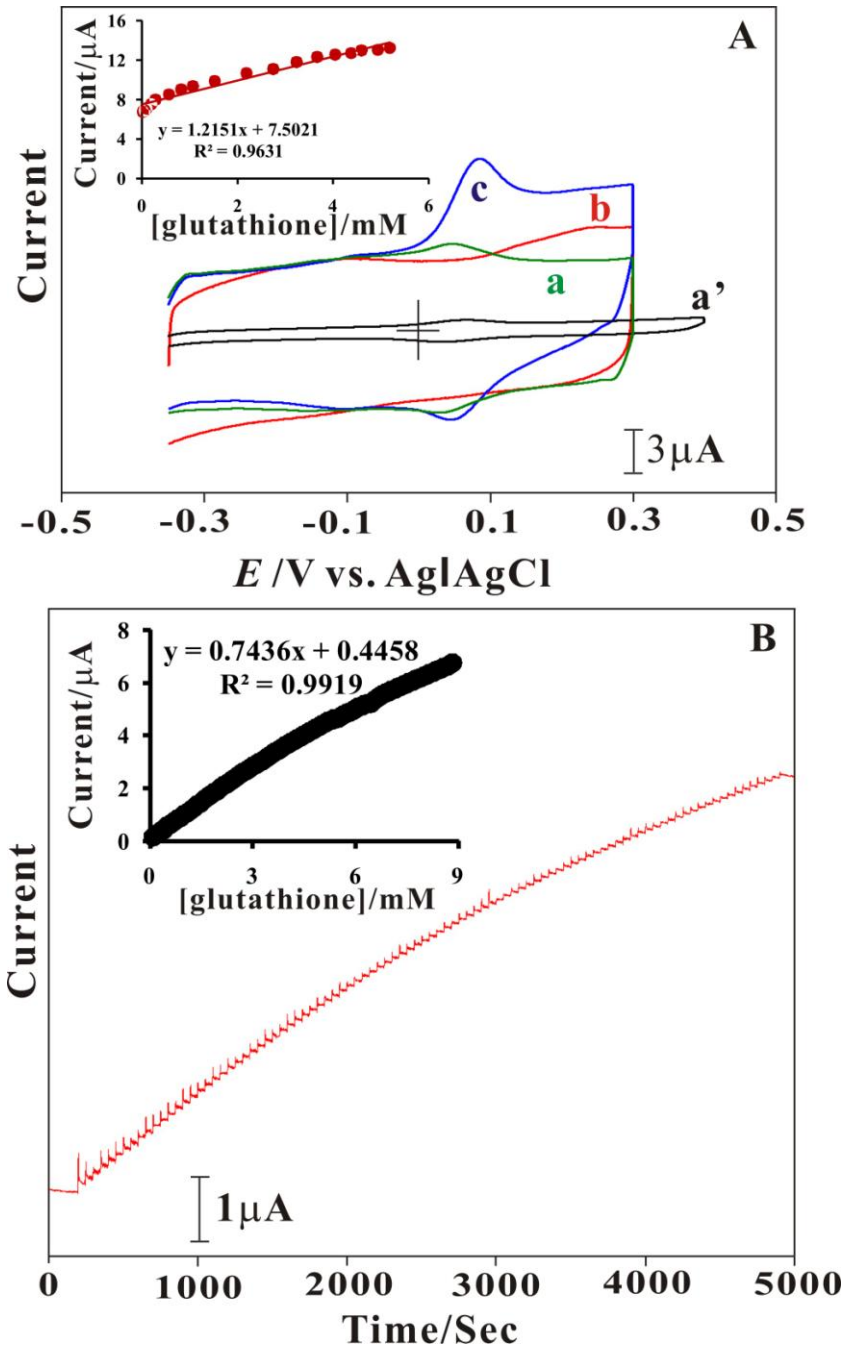

Figure 8: (A) Cyclic voltammogarms of (a') bare gold electrode; (b) only MWCNTs and (c) MWCNTs/GR in presence of 5.19 mM glutathione; (a) MWCNTs/GR absence in 0.1 M PBS (pH 7.0) at the scan rate of 100 mV s^{-1}, respectively. Insert shows different concentration vs. current. (B) Amperometric response to at MWCNTs/GR electrode to the successive injection of 100 μL of 0.1 M glutathione. Applied potential 0.08 V. Rotation rate: 1000 rpm. Insert shows different concentration vs. current.

Oxidation of reduced thiols (e.g. GSH) by DTNB was used (Eq. 1) to determine the concentration of total reduced thiol in a sample. The reaction involved electron transfer at the working electrode and thus the response was observed in amperometric mode [64-65].

$$2\ GSH + 5,5'\text{-dithiobis(2-nitrobenzoic acid)} \rightarrow GSSG + \text{2,2-nitro-5-thiobenzoic acid} \quad (1)$$

The oxidized form of glutathione (GSSG) is reduced by NADPH to the reduced form of glutathione (GSH) while NADPH is oxidized to NADP (Eq. 2). The reaction is catalyzed by the enzyme glutathione reductase (GR). The exchange of electrons at the working electrode gave rise to an amperometric response [66].

$$GSSG + 2\ NADPH + (GR) \rightarrow 2\ GSH + 2\ NADP + (GR) \quad (2)$$

Preparation of GSH/GSSG solution form GSH (10mM), DTNB (1.1 mg mL^{-1}) and NADPH (0.1 mg mL^{-1}) were dissolved in PBS (7.0) for electroctatlysis. Fig.8 (A) shows electrochemical of GSH at MWCNTs/GR modified electrode have been carried out using pH 7.0 (PBS) at 100 mVs^{-1} in the potential range of -3.5 to -0.3V . The cyclic voltammetric for MWCNTs/GR has exhibited a redox couple in the absence of the glutathione (curve a), upon addition of glutathione a new growth peak at 84 mV of respective analytes have appeared at the current values (curve c). An increase in concentration of glutathione, simultaneously produced a linear increase in the peak currents of the analytes with good film stability as shown in the inset. The detection limit concentration range for each analyte almost covers the concentration range found in the physiological conditions.

The observations at bare gold electrode (curve a') clearly indicate that the fouling effect of the electrode surface is the reason for obtaining the weak single peak for analytes. This result could also be explained in terms of higher diffusion rate of all the analytes at MWCNTs (curve b) when comparing MWCNTs/GR. However, the comparison of difference in I_{pc} values from Fig. 8 (A) obviously shows that the peak current of the analyte at MWCNTs/GR is higher than other electrodes, which reveals that GR redox couple involve and enhance the peak current of the analytes. From all these above results it is clear that MWCNTs/GR composite film is more efficient and exhibits enhanced functional properties comparing to that of MWCNTs alone.

3.5. Amperometric response of the analytes at MWCNTs/GR

In order to utilize the MWCNTs/GR have been synthesized on gold electrode for glutathione determination, amperometry under stirred condition was used in the further investigation to construct calibration curve. Figure 8 (B) shows amperograms obtained by holding the potential of MWCNTs/GR film electrode at 0.08 V and successive injection of 100 μL of 0.1 M glutathione to pH 7.0 PBS supporting electrolyte. For each addition, a well defined current response was obtained. As shown in figure 8 (B), the current in whole concentration range, 9.9×10^{-5} to 8.8×10^{-3} M. The sensitivity of MWCNTs/GR film electrode was found to be 6.2 μA mM^{-1} with a correlation coefficient of 0.9919.

The sensor achieves 98% of steady-state current in less than 5 s. Such a short response time indicates fast mass transfer across the film and also fast electron exchange between MWCNTs and analyte.

4. CONCLUSIONS

We have demonstrated application of MWCNTs/GR modified electrode for determination of glutathione. The modified electrode showed stable response. This feature provides a favorable clinical diagnosis for the electrocatalytic of glutathione at MWCNTs/GR electrode. High sensitivity and stability together with very easy preparation makes MWCNTs/GR electrode as promising candidate for constructing simple electrochemical sensor for glutathione determination.

The SEM and AFM results have shown the difference between MWCNTs, only GR and MWCNTs/GR films morphological data. Further, it has been found that the MWCNTs/GR has an excellent functional property along with good electrocatalytic activity on glutathione. The experimental methods of CVs and amperometric response with film biosensor integrated into the gold electrode and ITO which are presented in this paper, provide an opportunity for qualitative and quantitative characterization, even at physiologically relevant conditions. Therefore, this work establishes and illustrates, in principle and potential, a simple and novel approach for the development of a voltammetric and amperometric sensor which is based on the modified electrodes.

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