Journal of Biology and Today's World
ISSN 2322-3308
http://www.journalbio.com

Received: 23 August 2014 • Accepted: 12 November 2014

Research

doi:10.15412/J.JBTW.01040104

Design a biosensor for measuring H_2O_2 using modified carbon paste electrode with Single-wall Carbon Nanotubes and Catalase

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ABSTRACT

In this study, carbon paste electrode (CPE) was modified by single-wall carbon nanotubes (SWNTs) and catalase enzyme (CAT). The modified electrode was used for determination of hydrogen peroxide (H_2O_2). The SWNT was synthesized by catalytic chemical vapor deposition (CCVD) of methane on the Fe/Co/MgO catalyst and characterized using scanning electron microscopy (SEM), transmission electron microscopy (TEM), X-Ray Diffraction (XRD), Raman spectroscopy and UV-visible spectroscopy. The best experimental condition could result in carbon yield of 250% and high quality SWNTs with the controlled diameter of 0.9- 2.7 nm and length 5-30μm and purity more than 90% and less than 3% amorphous carbon were obtained. Direct electrochemistry of catalase enzyme in CPE was easily obtained and a reversible pair of peaks, that are similar, were appeared from Fe (II) and Fe (III) with the formal potential (E°) about +0.045 volts. This electrochemical process occurred in 0.1 M phosphate buffer solution (PBS) at PH=7. The designed sensor with high sensitivity and short response time (less than 8 second), and in the linearity of 3 to 370 μM can be used to determine the concentration of H_2O_2. Moreover, this sensor has a very good stability.

Key words: carbon paste electrode, single-wall carbon nanotubes, catalase enzyme, hydrogen peroxide

1. INTRODUCTION

Since the discovery of carbon nanotubes (CNTs) by Iizima in 1991, interest to exploit these miniaturized entities for using in such areas as field emitters, batteries, nanotube actuators , probe tips, reinforced composites, nanoelectronics display devices, sensors and biosensors etc. has been strongly increased. CNTs can be created using chemical vapor deposition, carbon arc methods, Ball milling or laser evaporation. Single-walled carbon nanotubes (SWNT) and multi-walled carbon nanotubes (MWNT), as the two main variants, have a high tensile strength (Young's modulus > 1 terapascal, making CNTs as stiff as diamond and flexible along the axis), ultra-light weight, and excellent chemical and thermal stability. They are known for having semi- and metallic-conductive properties (1). These features has led to many suggested applications in the biomedical field, including biosensors, drug and vaccine delivery. Both SWNTs and MWNTs are typically a few nanometers in diameter and several micrometers (10μm) to centimeters long. SWNT possesses a cylindrical nanostructure with high aspect ratio that is formed by a rolling up single graphite sheet called graphene into a tube form. All the carbons in graphene sheets are sp2 hybridized and have a delocalized π electron structure that is responsible for the extraordinary electronic qualities. CNT displays metallic, semiconducting and

superconducting electron transport, has a hollow core suitable to store guest molecules and has the largest elastic modulus of any known material (2). Biosensors, as electronic devices, through using a biological element, can yield quantitative or semi-quantitative analytical information. In the two last decades, biosensors, owing to their smart size and quick and dependable response compared to the conventional systems, have been finding numerous attentions to be applied in the field of clinical diagnosis, drug discovery, detection of environmental pollutants, biotechnology, and military and civil defense. A biosensor has three parts including: (1) biological detection element that recognizes the substance of interest, (2) transducer that converts a biorecognition event into measurable signal and (3) a signal processing system that converts the output into a processable signal. Biological detection elements include enzymes, antibodies, tissue slices, DNA or cell membrane receptors and so forth. The biological molecules are naturally immobilized in close proximity to a transducer surface thereby facilitate direct or mediated signal transfer to the transducer. The effectiveness of a biosensor depends generally on the effective electron transfer, stability of the biomolecule, reusability, linearity and sensitivity, etc. It has been investigated that transduction efficiency, as a key factor, yields information on analytical properties of sensor such as signal stability, reproducibility, detection limit, and in some particular cases operational stability and selectivity. Enzyme electrodes bring together the specificity of enzymes with the analytical power of electrochemical devices, and are strongly useful for clinical diagnostics or environmental monitoring. Catalase is a heme protein in the group of oxidoreductases with ferriprotoporphyrin-IX at the redox center, and catalyzes the disproportionation reaction of hydrogen peroxide. Similar to other enzymes, it is difficult for catalase to transfer electrons, since they are structurally big and complex, where the redox centers profoundly immerse in the bodies. CNTs modified electrodes boost the electrical contact between the electrode and the catalase. The application of CNTs to biosensors has extremely increased stability of the immobilized enzymes resulting in enhanced biosensor response. Moreover, the CNT modified biosensor displays an enhanced stability and approximately eight-fold sensitivity (3). Hydrogen peroxide is the most important by-product of many enzyme catalyzed reactions based on glucose oxidase, choline oxidase, alcohol oxidase, cholesterol oxidase and lactate oxidase etc. It is also an essential factor in food, commercial cultivation of various kinds of eatable fungi and algae, pharmaceutical and in environmental and medical applications. The catalase/CNT modified gold electrode was reported by Zhou and coworkers (4). Salimi et al have indicated direct electron transfer in catalase immobilized MWCNT biosensor for a pair of welldefined and nearly reversible cyclic voltammetry peaks for Fe (III)/Fe (II) redox couple of catalase. The high value of Michaelis–Menten constant (1.70 mM) indicates potential applicability of the electrode as a reagentless biosensor based on the direct electrochemistry of the catalase enzyme (5). Xu et al. (6) developed a hydrogen peroxide biosensor based on the attractive features of the carbon nanotube. The device relied on the coimmobilization of HRP and the methylene blue (7) mediator on the CNT-coated electrode, with MB functioning as electron carrier between the enzyme and the surface. The response to hydrogen peroxide was linear up to 2 mM and had a detection limit of 1 μM. Zhou et al. (8) reported on the direct electrochemistry of catalase at SWCNT-modified gold electrodes. A well-defined redox process corresponding to the Fe (III)/Fe (II) redox center was observed around -0.4 V. The peak current increases with the enzyme concentration over the micromolar range. The catalase/SWCNT modified electrode displayed a characteristic catalytic wave upon addition of H_2O_2.Our aim is to design a more efficient and sustainable biosensor, with a higher sensitivity and precision, as well as affordable if commercialized.

2. MATERIALS AND METHODS

2.1. Apparatus

Cyclic voltammetric (CV) experiments were performed using a Dutch Palm-Sense potential-galvanometer, equipped by a personal computer. A conventional three-electrode cell was used throughout the experiments, with bare or SWNTs modified CPE as a working electrode, a SCE as a reference electrode and a platinum electrode as a counter electrode. Microscopic images of SEM and TEM were taken using Zeiss Company microscopes model DSM 960A and CEM 902A. XRD was measured using the instrument of Germany's Siemens and CuKα was obtained 1.54056 A˚. Visible-Ultraviolet spectroscopy was obtained by Shimadzu UV 160 Spectrophotometer. Raman spectroscopy was obtained using Almega Roman Spectrometer and at room temperature. A fixed bed flow and a ceramic boat (length, wide, height: 1.5×1×8) were used for SWNTs preparation.

2.2. Materials

$Fe(NO_3)_3 .9H_2O$, $Co(NO_3)_2 .6H_2O$, MgO, bovine catalase enzyme (40–45 units/mg), graphite powder, paraffin wax, phosphate buffer solution (PBS) including sodium phosphate solution (NaH_2PO_4 and Na_2HPO_4), H_2O_2, ethanol, H_2SO_4 98%, HNO_3, HCL 3 N, dimethyl-formamide (DMF) and 1,3-dicyclocarbodiimide (DCC). All solutions and materials were purchased from Sigma-Aldrich Company. All solutions were made using double-distilled water.

2.3. Synthesis of SWNTs

In this project, SWNTs along with Fe/Co metal catalyst were grown on MgO basis using CCVD. Then, grown nanotubes were putrefied in two processes of under atmospheric oxidation and acidic stew with HCL. SWNTs

synthesis through this method consists of two stages: 1) catalyst preparation, and 2) SWNTs synthesis. Bimetallic catalyst of Fe/Co was prepared using wet chemical saturation method on MgO basis. 500 mg MgO was dispersed in 10 ml distilled water and the resulting suspension for 60 min in an ultrasonic device was changed into homogeneous suspension. Metallic nitrates of $Fe(NO_3)_3.9H_2O$ and $Co(NO_3)_2.6H_2O$ with the ratios of Fe/Co/MgO : 15/15/70 were added to suspension and exposed to ultrasonic for 30 minutes. Then, this mixture was dried at the temperature of 120°C and milled to a soft powder. Next, the obtained catalyst was use as a basis for the growth of nanotubes.

2.4. The growth of SWNTs

First of all, the catalyst was revived using hydrogen gas and in the temperature of 500-600ºC. To this end, the prepared catalyst was spread into a ceramic boat, was placed inside a fixed bed flow, and after being revived by hydrogen gas the gas flow was interrupted, and nitrogen gas with volumetric flow rate of 150ml/min and the temperature of 800ºC was remained for 30 minutes. To grow SWNTs, methane gas with volumetric flow rate of 30ml/min and the temperature of 870ºC was entered into the system for 60 minutes. Finally, methane gas flowing was stopped and the boat's product under a nitrogen flow was cooled to room temperature (9). In this project, high purity (99%) hydrogen, nitrogen, and methane gases were used.

2.5. SWNTs purification

Synthesized nanotubes, in order to be purified, were at first oxidized for 60 minutes in the temperature of 400º to remove amorphous carbon. In order to separate MgO and metallic nanoparticles of Fe and Co, 200 mg of the sample underwent acidic stew in 50ml HCL 3N for 6 hours. After separating purified SWNTs and washing them with distilled water, in order to remove the acidic effect, the nanotubes were dried at 100ºC for 24 hours (10-12). Structural and morphological analysis of the samples was done using SEM, TEM, XRD, Raman spectroscopy and UV-Visible spectroscopy analytical methods.

2.6. Activation of SWNTs

SWNTs were activated using the method introduced by Louis et al (13). According to this method, 2 mg SWNTs were added to 10 ml saturated H_2SO_4 and HNO_3 with the ratio of 3:1. This solution was placed for 4 hours in sonication bath with the power of 55 Hz. Then, nanotubes were washed using deionized distilled water, and were collected by a 0.45 µm diameter filter. This process continues till the water used in washing has a neutral pH. In this method, by oxidizing SWNTs through using acid, formation of Co_2 gas and its getting out of the solution, activated carboxyl groups are formed at the end of the SWNTs, and thus, these nanotubes get activated.

2.7. Preparation of Working Electrode

To prepare carbon paste, graphite powder and paraffin wax were mingled within a watch glass with the ratio of 7:3, and were homogenized at 80ºC until a completely smooth paste was obtained. In order to prepare modified electrode, some part of this paste along with SWNTs and catalase enzyme were pressed into a cylindrical container. Electrical connection was created by a copper wire connected to the bottom of the container. Some of the carbon paste was stored in order to be used in comparing voltammogram charts with the modified electrode. Working electrode was prepared in two steps. In the first step the SWNTs were attached to the working electrode. This step is done through incubation of the electrode in a solution of SWNT/DMF, and in the presence of 0.5 mg/ml 1-3 dicyclocarbodiimide (DCC). Doing this, carboxyl groups created at the end of carbon nanotubes are converted into amide groups and the connection of nanotubes on the electrode surface and catalase enzyme will be done more simply and accurately (Figure 1).

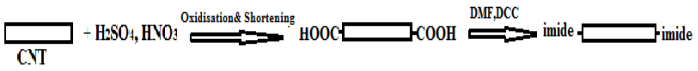

Figure 1. Preparation of aligned CNTs functionalized with biomolecules

As a result, SWNTs, which owing to the carboxyl groups have an activated end, are connected to the working electrode. SWNT/DMF solution with sonication of 1 mg SWNTs was prepared in 1 ml DMF solvent in 3-5 minutes was seeped on electrode surface. Before DMF solvent is completely evaporated, the second step (connecting the catalase enzyme to the modified electrode) was performed by adding the catalase enzyme to the modified electrode. At this stage, the catalase enzyme with concentration of 2.5 mg/ml was solve in 25 mm sodium phosphate buffer, and at 25ºC, was added to the modified electrode and incubated until it was completely dry. Through washing the electrode surface with DMF solvent, phosphate buffer (pH=7.4), and finally with deionized water, the catalase enzyme which did not enter into the reaction was eliminated. Working electrode was completely dried in room temperature and was polished by dragging on a quite smooth surface (Figure 2).

Figure 2. CNTs nanoelectrode

3. RESULTS AND DISCUSSION

3.1. Examining the properties of SWNTs by electron microscopy

SEM images of SWNTs, before and after purification, grown on MgO catalyst, in which the Fe/Co catalyst is used, are shown in figure 3. As it is shown in the A part of the Figure 3, synthesized nanotubes, before purification, have various impurities such as catalyst materials and other carbon forms. After the oxidation step and acidic operation the resolution of SEM images are increased, which confirms the fact that the purification process has been effective (part B of figure 3).

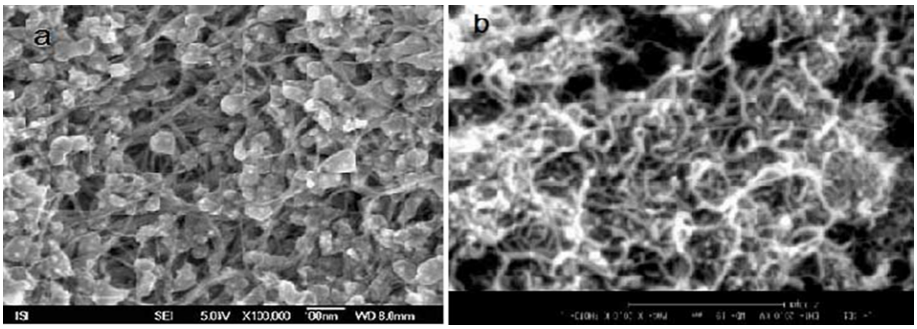

Figure 3. SEM image of SWNTs synthesized on MgO basis on Fe/Co catalyst: A) before purification; B) after the two-step purification

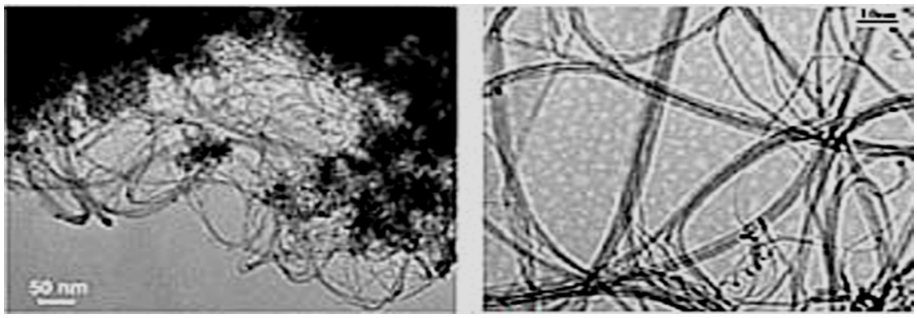

Figure 4. TEM images of synthesized SWNTs: A) initial SWNTs; B) SWNTs after the final purification

TEM images of synthesized SWNTs are shown in Figure 4. According to information taken from the TEM and SEM images, the synthesized nanotubes have a diameter of 0.9-2.7 nm, length of 5–30 μm, more than 90% purification, and less than 3% amorphous carbon. Figure 5 shown SEM images of Bare CPE, CPE modified by SWNTs and CPE modified by SWNTs and catalase enzyme.

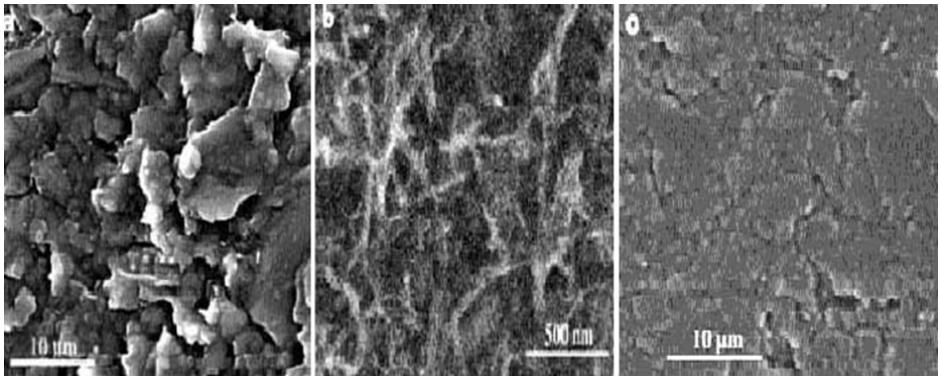

Figure 5. SEM images: a) Bare CPE; b) CPE modified by SWNTs; c) CPE modified by SWNTs and catalase enzyme

3.2. UV-Visible spectrum characteristics of synthesized nanotubes

UV-Visible spectroscopy is a useful technique for determining the amount of the SWNTs functionalizing and examining how nanotubes are dispersed in aqueous environment. Free л electron in initial nanotubes has a special absorption in UV-Visible spectrum. These nanotubes have a special peak in the wavelength of 170nm (14-17) (Figure 6).

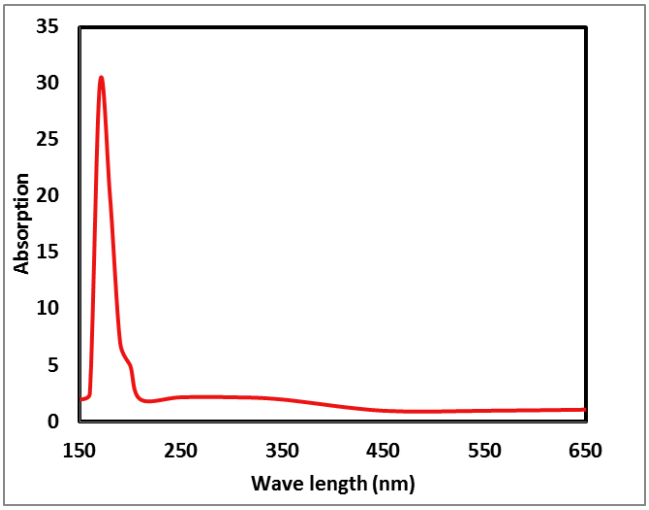

Figure 6. UV-Visible spectroscopy of synthesized SWNTs after purification

As a result of SWNTs activation, a particular peak is observable in the wavelength of 230nm. The peak observed in the wavelength of 230 nm is due to the SWNTs carboxylation (Figure 7). This peak is created because of the two free electrons of -c=o existed in

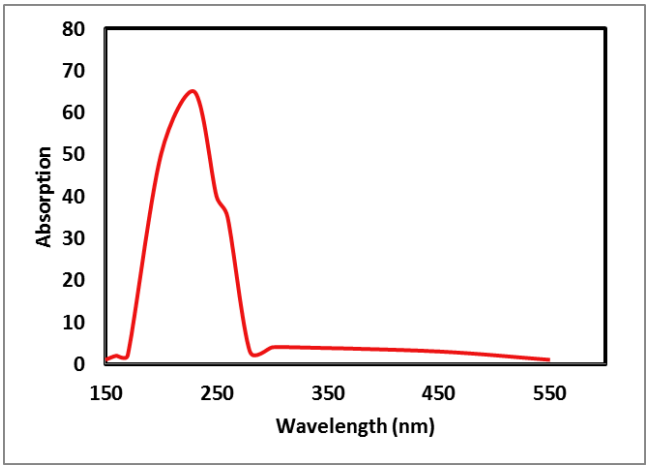

Figure 7. UV-Visible spectroscopy of synthesized SWNTs after activation

UV-Visible spectrum of synthesized SWNTs, after purification and functionalizing, indicates the high quality of produced nanotubes (there is a sharp peak at a wavelength of 170nm), and the good functionalization of the nanotubes.

3.3. Raman spectroscopy of synthesized SWNTs

For specifying the detailed combination of chiralities in the SWNTs material and assessing purity, Raman spectroscopy has been widely used (Figure 8). There are three Raman spectrum areas of primary interest for SWNTs. The radial breath mode (RBM) from about 120 to 300 cm^{-1} is unique to SWNTs and is used to determine tube diameter. Several lasers of different excitation frequencies should be used so that a comprehensive image of the chiralities can be obtained. In the Raman spectrum of SWNTs, two additional bands – the D band at ~ 1350 cm^{-1} and the G band at 1500 to 1586 cm^{-1} – can be observed. (18).The ratio of the height of the G band to that of the D band has been widely used as a measure of SWNTs purity. Figure 8 shows the Raman spectra of synthesized nanotubes. The (a) part of figure 8 shows the initial nanotubes. As it can be seen, the ratio of peak D to peak G and the under-curve graph show the impurities and abundant amorphous carbon before purification and activation of carbon nanotubes. The (b) part of the figure shows the carbon nanotubes after purification and activation. Dramatic reduction of the peak D compared with peak G reflects the effectiveness of the operation (19).

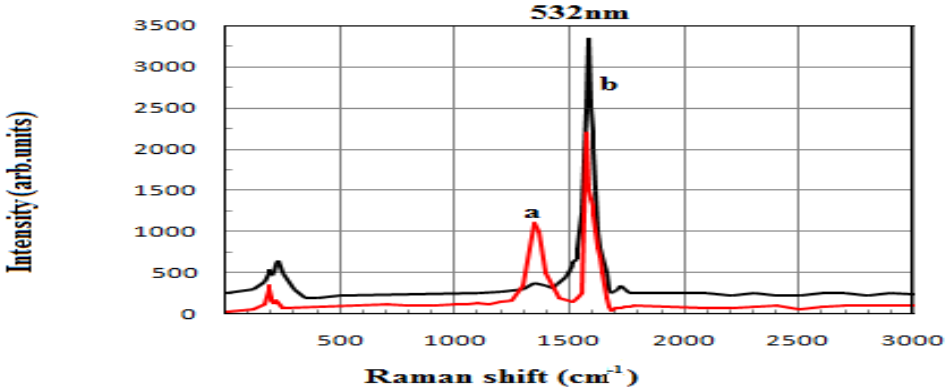

Figure 8. Raman spectroscopy of synthesized SWNTs :A)) initial SWNTs; B) SWNTs after the final purification

3.4. X-Rey diffraction pattern of synthesized SWNTs

For further exploration of the properties of synthesized crystalline nanotubes, XRD pattern was used that is shown in Figure 9.

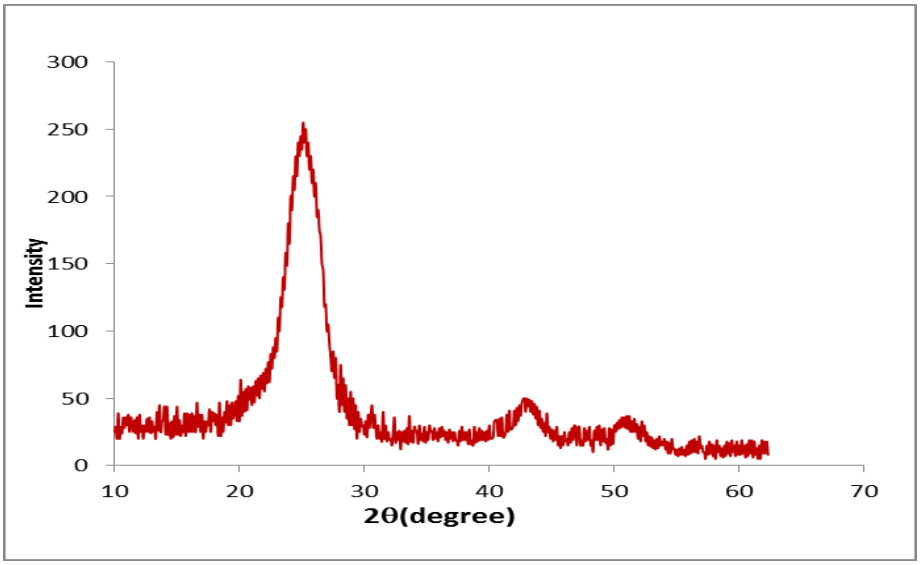

Figure 9. X-Rey diffraction pattern of synthesized SWNTs

As shown in figure 9, the peak at 2θ=25.1˚is related to the SWNTs, and the peak at the angles 43º and 52º is related to MgO and Fe/Co. As the figure 9 shows, the synthesized nanotubes, after purification, have small amounts of metal catalysts.

3.5. Direct electrochemistry of CAT/SWNTs/CPE

Using CV, the electrochemical behaviors of CAT / SWNTs/ CPE were assessed the result of which is given in Figure 10. The CV comparison of (a) SWNT/CAT/CPE, (b) SWNT/CPE, (c) CAT/CPE, and (d) bare CPE in 0.1 M PBS, with pH=7 and the scan rate of 100mV, are respectively demonstrated in figure 10. In part (a) a pair of redox peak can be observed with the Epc=0 V and Epa=+0.09 V (vs.SCE). The formal potential (E˚′), calculated by the midpoint of Epa and Epc, was obtained as +0.045 V (vs. SCE). In parts (b), (c) and (d) no voltammetric response was observed. According to the results, the CPE modified by SWNTs and catalase enzyme provided the catalase enzyme molecules with a suitable microenvironment and the presence of SWNTs in the electrode could boost the electron transfer rate for catalase enzyme. Then, the direct electron transfer of catalase enzyme in the modified CPE was achieved successfully.

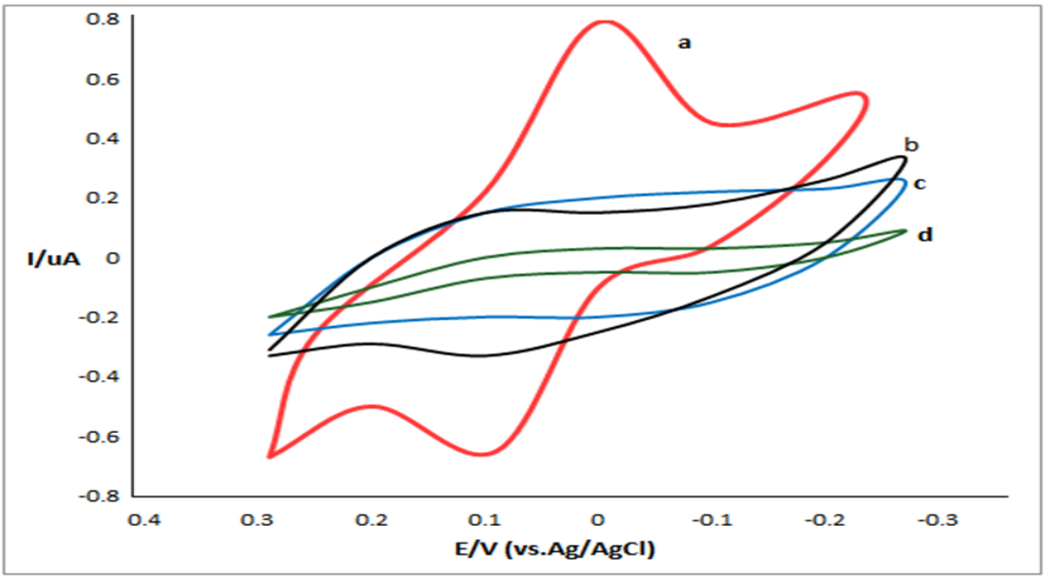

Figure 10. CV of (a) CAT/ SWNTs/ CPE (b) SWNT/CPE (c) CAT/CPE (d) bare CPE in 0.1 M PBS, pH=7 and scan rate of 100mV/sec

In the next part of the study, the electron transfer properties of catalase enzyme on modified CPE with SWNTs were investigated and the effect of different scanning rates on a cyclic voltammogram of catalase enzyme was evaluated. In Figure 11, the linear range between the anodic and cathodic flow of catalase with different scanning rates is observed. Also Dependence of the anodic and cathodic peak currents on the scan rates showed in Figure 12.

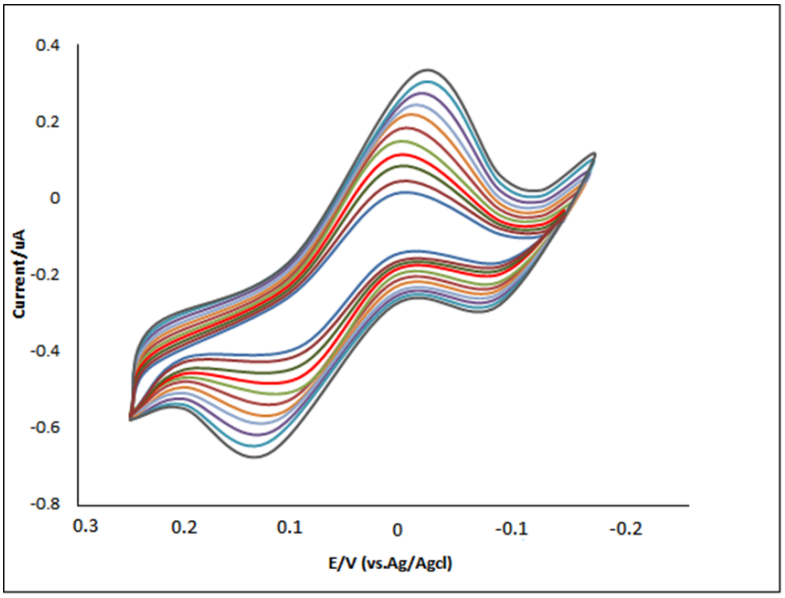

Figure 11. Typical cyclic voltammograms of CAT/SWNTs/CPE at scanning rates of 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 mV/sec. (at pH=7 and in 0.1M PBS)

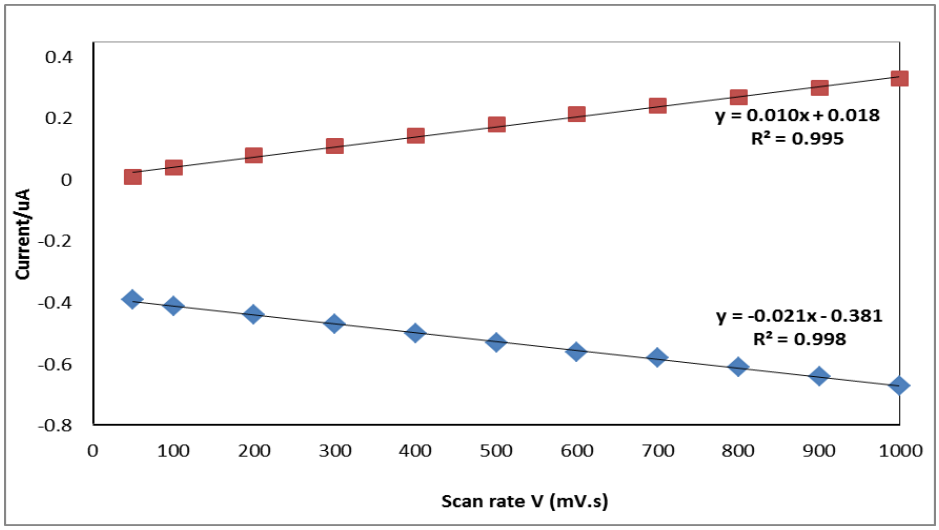

Figure 12. Dependence of the anodic and cathodic peak currents on the scan rates. All the data were obtained at pH=7 and in 0.1M PBS

Correlation coefficient for the cathodic and anodic peaks was calculated respectively as 0.995 and 0.998. This phenomenon shows that redox process is controlled by the absorption on the electrode surface and confirms the stable consolidation of catalase on the electrode surface. However, there is clearly a systematic deviation from linearity in this data. Low scan rates are always on one side of the line and the high scan rate points are on the other. The anodic and cathodic peak potentials are linearly dependent on the logarithm of the scan rates (ν) when ν > 1.0 V/s, which is in line with the Laviron theory, with slopes of -2.3RT/αnF and 2.3RT/ (1-α) nF for the cathodic and the anodic peak, respectively (20). The following equation was used to calculate the heterogeneous electron-transfer rate constant (ks) (21-23):

$$[\log ks = \alpha \log(1-\alpha) + (1-\alpha)\log\alpha - \log\frac{RT}{nF\nu} - \frac{\alpha(1-\alpha)nF\Delta EP}{2.3RT}] \quad (1)$$

In this equation, **n** is the number of transferred electrons at the rate of determining reaction and R, T and F symbols having their conventional meanings. In order to obtain electrochemical parameters, the relationship between the peak potential and Lnν is calculated. Moreover, two linear regression equations are obtained. The slope and intercept of the obtained lines, α=0.34, n=0.97, and Ks has been calculated as 1.012 S^{-1}.

3.6. Effect of pH Solution on direct electron transfer of Catalase enzyme

In order to obtain an efficient biosensor for H_2O_2, the influence of pH on the response of CAT/SWNTs/CPE was investigated. It was observed that the variations of peak potentials of cyclic voltammogram and the currents are reversible by pH in range of 5 to 7; that means, if the electrode is transferred from a solution with different amounts of pH into the initial solution, a similar cyclic voltammogram is obtained. An increase in the solution pH will lead to a shift in the position of the cathodic and anodic peak potentials. The graph of potential against pH (5 to 11) creates a line (Figure 13) which has a correlation coefficient of 0.997 indicating the linear relationship between the potential of the aforementioned electrode and pH of 5 to 11.

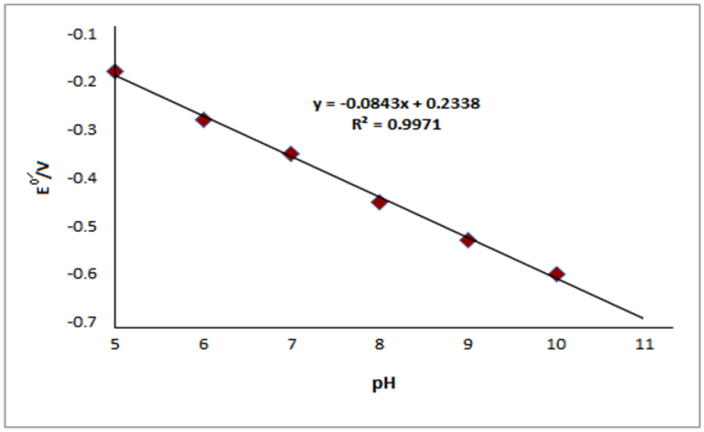

Figure 13. Dependence of the current response of CAT/SWNTs/CPE electrode to 30µM H_2O_2 on the pH of buffer solution

3.7. Using CAT/SWCNs/CPE in determining H_2O_2 concentration and biosensor design

Using differential pulse voltammetry (DPV) technique, the detection limit of H_2O_2 was determined. Compared with CV, this technique has a better current sensitivity and resolution that is why it is used to estimate a lower detection limit. Adding H_2O_2 in 0.1M PBS and pH of 7, the cyclic voltammogram of the working electrode for the direct electron transfer of catalase enzyme changed significantly with an increase of reduction peak current and a decrease of oxidation peak current (Figure 14); however, the cyclic voltammogram change of bare or SWNTs/CPE was insignificant (not shown), implying an obvious electrocatalytic behaviour of the catalase enzyme to the reduction of H_2O_2.The electro-catalytic process can be expressed as follows:

$$CAT\text{-}Fe\ (III) + H_2O_2 \rightarrow Compound\ I + H_2O \quad (2)$$

$$Compound\ I + H_2O_2 \rightarrow CAT\text{-}Fe\ (III) + O_2 + H_2O \quad (3)$$

$$CAT\text{-}Fe\ (III + H^+ + e^- \rightarrow CAT\text{-}Fe\ (II)\ (at\ electrode) \quad (4)$$

$$CAT\text{-}Fe\ (II) + O_2 \rightarrow CAT\text{-}Fe\ (II)\text{–}O_2\ (fast) \quad (5)$$

$$CAT\text{-}Fe\ (II)\text{–}O_2 + 2\ H^+ + 2\ e^- \rightarrow CAT\text{-}Fe\ (II) + H_2O_2\ (at\ electrode) \quad (6)$$

Calibration curve (Figure 15) indicated the linear dependence of the cathodic peak current on the H_2O_2 concentration in the range of 3 to 370 µM. In Figure 14, at higher concentration of H_2O_2, the cathodic peak current decreased and remains constant. This implies electrocatalytic property of electrode. Thus, this experiment has introduced a new biosensor for the sensitive determination of H_2O_2 in solution.

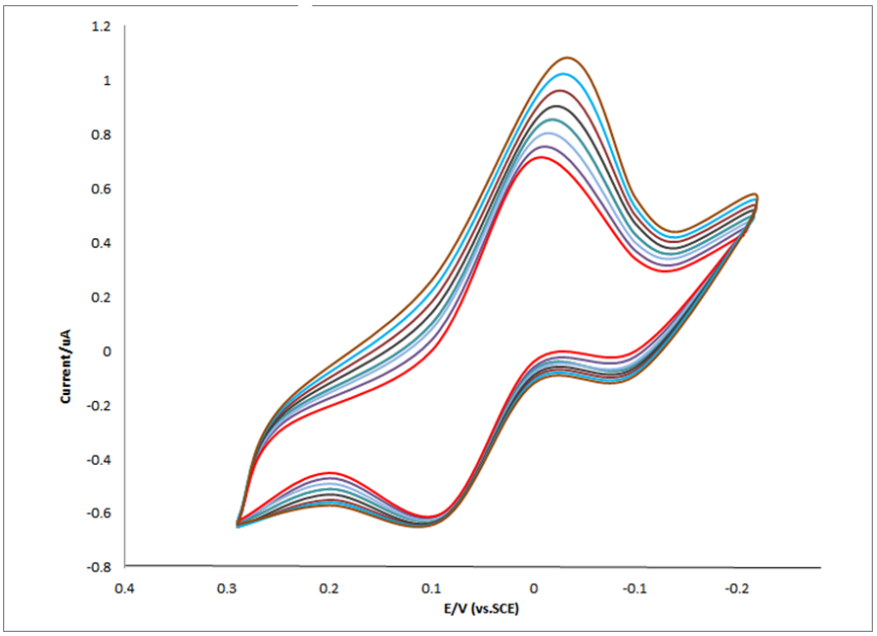

Figure 14. Cyclic voltammogram of CAT/ SWNTs/CPE in different concentrations of H_2O_2 and in 0.1 M buffer and pH=7

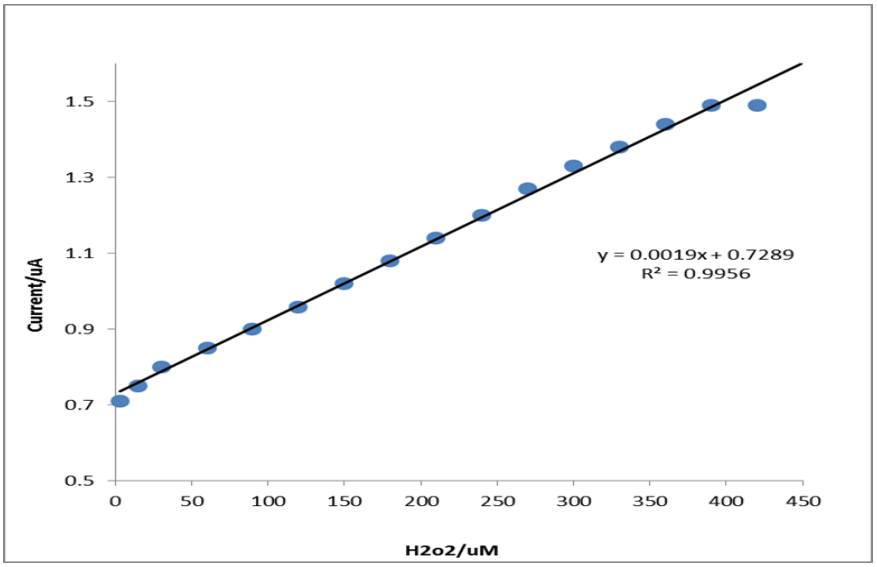

Figure 15. Relationship between cathodic peak current of catalase enzyme and different concentrations of H_2O_2 in the concentration range of 3 to 370 µM (scan rate: 100 mV/sec)

3.8. Stability of H_2O_2 biosensor

For determining the stability of CAT/SWNTs/CPE electrode, the biosensor was placed at the temperature of 4ºC for 26 days and it was observed that the sensor's 84% of activity was retained. A constant current also could be maintained while 180 cycles were repeated in the presence of H_2O_2. This sensor can be reproduced when the catalase solution is dropped on its surface, after rubbing the electrode tip gently on a clean paper and then on a smooth glass. The respond of new surface current in the concentration (50µM) H_2O_2 was tested. The relative standard deviation for seven consecutive repetitions was obtained as 3.8%. In addition, this sensor demonstrated the ability of reproducing six-electrode, separate producing and acceptable repeatability with standard deviation of 4.5% for the determined current. The response time of the sensor was obtained as less than 8 seconds in repeated measurements.

4. CONCLUSION

Catalase enzyme can be effectively immobilized in a SWNTs/ CPE electrode. The CAT/SWNTs/CPE electrode shows a fast direct electron transfer of CAT. Direct electron transfer of the CAT/SWNTs/ CPE electrode was easily achieved. The use of SWNTs in this sensor reduces the response time and increases the linear range of H_2O_2 measurement compared with the sensors designed by nanoparticles, promising a wider use of nanotubes in designing sensors.Therefore, this research is considered an efficient strategy that introduces a new biosensor for the study of electron transfer.

ACKNOWLEDGMENT

For his many helpful and constructive grammatical and linguistic comments, we would like to express our endless gratitude and indebtedness to Mr. Roohollah Datli Beigi, M.A. in English Language and Literature, University of Isfahan.

AUTHORS CONTRIBUTION

This work was carried out in collaboration among all authors.

CONFLICT OF INTEREST

The authors declared no potential conflicts of interests with respect to the authorship and/or publication of this article.

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