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Direct Electron Transfer of Cytochrome c on ZrO_2 Nanoparticles Modified Glassy Carbon Electrode

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In this study we used of nanotechnology, electrochemistry and biology for investigation of direct electron transfer of cytochrome c (cyt c) on ZrO_2 nanoparticles (Nps) modified glassy carbon electrode (GCE). Properties of Synthesized ZrO_2 nanoparticles were investigated by X-ray diffraction (XRD) and UV-visible devices. Direct electrochemistry of the modified GCE with Cyt c and ZrO_2 nanoparticles achieved and formal potential (E^0) calculated as: -117.5 mV versus reference electrode. Protein structure of cytochrome c with heme center could change oxidative iron state (Fe^{3+}) to reductive state (Fe^{2+}) and vice versa by electron transfer. We used of this electron transfer in designing a biosensor for hydrogen peroxide (H_2O_2) detection. The linear dependence of cathodic peak on the H_2O_2 concentration was observed in the range of 40 to 270µM; also designed biosensor showed a good reproducibility and stability.

Keywords: Bioelectrochemistry, Cytochrome c, ZrO_2 Nps, Glassy Carbon Electrode

1. INTRODUCTION

Nanotechnology, the manipulation of matter on a near-atomic scale to produce new structures, materials, and devices offers the promise of unprecedented scientific advancement for many sectors, such as medicine, consumer products, energy, materials, and manufacturing [1-3]. Nanotechnology has the power not only to improve existing technologies, but to dramatically enhance the effectiveness of new applications [4-5]. Research on the potential applications of nanotechnology continues to expand

rapidly worldwide. New nanotechnology consumer products emerge at a rate of three to four per week [6-7]. Nanotechnology has the potential to dramatically improve the effectiveness of a number of existing consumer and industrial products. Over the last decade several developments in electrochemistry have contributed significantly to nanoscience and nanotechnology [8]. Most important are the advances in nanoscale characterization of electrochemical interfaces described above, progress in electrochemical processing methods for the formation of micro- and nanostructures, and the discovery of electrochemical techniques and concepts by the nanotechnology community, in particular for studying functional nanostructures[9-11]. Atomic-scale dynamic processes on electrode surfaces, such as surface diffusion, interactions between adsorbates, or the initial stages of nucleation, are of considerable importance to a wide variety of electrochemical reactions. Detection of very small amounts of a chemical structure in medical, industrial and biological systems are envisioned from the integration of chemical, physical and biological devices working together as an integrated sensor at the nanoscale[12]. Analytical chemistry plays an important role in our everyday life because almost every sector of industry and public service relies on quality control [13-15]. The majorities of chemical analysis methods is time-consuming and heavily employ expensive reagents and equipment in order to achieve high selectivity and low detection limits [16]. The bioanalytical nanosensors either use biology as a part of the sensor, or are used for biological samples. An electrochemical nanobiosensor is a self-contained integrated device, which is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor) which is retained in direct spatial contact with an electrochemical transduction element [17-19]. In this study we investigated direct electron transfer of cytochrome c on ZrO_2 nanoparticles modified glassy carbon electrode. Our research led to design a new biosensor for detection of hydrogen peroxide (H_2O_2). Cytochrome c that used in our research as heme-protein structure is a component of the electron transport chain in mitochondria and also is an essential component of the electron transport chain, where it carries one electron [20-21]. It is capable of undergoing oxidation and reduction, but does not bind oxygen. The molecular structure of cytochrome c was shown in figure 1. The heme group is clear in centre of protein structure. This heme group played an important role in reduction (Fe^{2+}) an oxidation (Fe^{3+}) state in our electrochemical experiments [22].

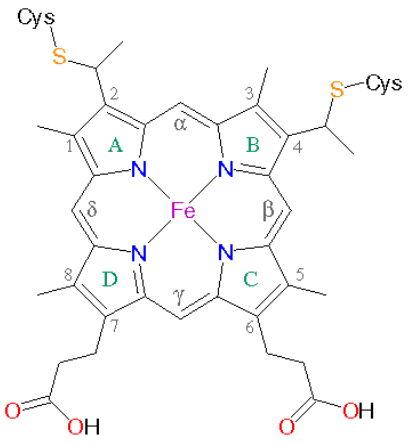

Figure 1. Molecular structure of cytochrome c

A bulk material should have constant physical properties regardless of its size, but at the nano-scale size-dependent properties are often observed. Thus, the properties of materials change as their size approaches the nanoscale and as the percentage of atoms at the surface of a material becomes significant [23]. The interesting and sometimes unexpected properties of nanoparticles are therefore largely due to the large surface area of the material, which dominates the contributions made by the small bulk of the material [24-25]. Zirconia (ZrO_2) Nanoparticles was used in this research as Facilitator electron transfer between cytochrome c and glassy carbon electrode. Glassy carbon is widely used as an electrode material in electrochemistry, as well as for high temperature crucibles and as a component of some prosthetic devices, and can be fabricated as different shapes, sizes and sections [26-27]. After studies about direct electron transfer of cytochrome c on ZrO_2 nanoparticles modified glassy carbon electrode, we designed a biosensor for hydrogen peroxide detection (H_2O_2). Hydrogen peroxide is a reactive oxygen metabolic by-product that serves as a key regulator for a number of oxidative stress-related states [28-30]. It is involved in a number of biological events and intracellular pathways that have been linked to several diseases [31-34]. Measurements of reactive oxygen species help to determine how oxidative stress modulates varied intracellular pathways. We hope with this designed biosensor, opened a new way for hydrogen peroxide detection that this detection is very important in animal& plant physiological activities.

2. EXPERIMENTAL

2.1. Materials

Cytochrome c and Zirconyl chloride octahydrate purchased from Sigma-Aldrich. Other Reagents purchased from Merck. The supporting electrolyte used for all experiments was 0.1 M pH 7 phosphate buffer solution (PBS), which prepared by using 0.1 M Na_2HPO_4 and NaH_2PO_4 solutions. All the reagents used were of analytical grade and all aqueous solutions were prepared using doubly distilled water generated by a Barnstead water system.

2.2. Apparatus

Cyclic voltammetry (CV) and square wave voltammetry were performed using an Autolab potentiostat PGSTAT 302 (Eco Chemie, Utrecht, The Netherlands) driven by the General purpose Electrochemical systems data processing software (GPES, software version 4.9, Eco Chemie). A conventional three-electrode cell was employed throughout the experiments, with bare or ZrO_2 nanoparticles modified glassy carbon electrode (3.0mm diameter) as a working electrode, a saturated calomel electrode (SCE) as a reference electrode, and a platinum electrode as a counter electrode. The phase characterization of ZrO_2 nanoparticles was performed by means of X-ray diffraction (XRD) using a D/Max-RA diffractometer with Cu$K\alpha$ radiation. The absorbance properties of prepared nanoparticles were measured and recorded by using a TU-1901 double-beam UV-visible spectrophotometer.

2.3. Preparation of ZrO_2 nanoparticles

The ZrO_2 nanoparticles were prepared according to the literature. Initially, 2.58 g $ZrOCl_2 \cdot 8H_2O$ and 4.80 g urea were dissolved in 20.0 mL CH_3OH under stirring to form a colorless solution. The solution was transferred to a 20-mL Teflon-lined stainless steel autoclave, which was heated to 200 °C and maintained at that temperature for 20 h. The obtained white product was post-treated with sulphuric acid solution (0.16 mmol), and then calcined at 645 °C.

2.4. Preparation of unmodified glassy carbon electrode

The most commonly used carbon-based electrode in the analytical laboratory is glassy carbon (GC). It is made by pyrolyzing (Pyrolysis is the decomposition of organic compounds by heating to high temperatures in the absence of oxygen) a carbon polymer, under carefully controlled conditions, to a high temperature like 2000◦C [35]. An intertwining ribbon-like material results with retention of high conductivity, hardness, and inertness. Glassy carbon electrode (GCE, dia. 3mm) was polished with 1 μm and 0.05 μm alumina slurries sequentially and then rinsed with distilled water. After that, the electrode was sonicated in deionized water and finally dried under ambient conditions.

2.4. Preparation of modified glassy carbon electrode with ZrO_2 Nanoparticles and Cyt c

To prepare the modified GCE with ZrO_2 Nanoparticles and Cyt c, after immobilized ZrO_2 Nanoparticles on GCE surface, The ZrO_2 Nanoparticles /glassy carbon electrode was placed into a fresh PBS including 10mg mL^{-1} Cyt c (pH 7.0, 3 to 5°C) for 8 hour. At the end, the modified electrode was washed with deionized water and placed in PBS (PH 7.0) at a refrigerator (3 to 5°C), before being employed in the electrochemical measurements as the working electrode.

3. RESULTS AND DISCUSSION

3.1. X-Ray diffraction of ZrO_2 nanoparticles

The XRD pattern Fig. 2 for ZrO_2 nanoparticles, the diffraction peaks are absorbed at 2θ values. The prominent peaks have been utilized to estimate the grain size of sample with the help of Scherrer equation [36] $D = K\lambda/(\beta \cos \theta)$ where K is constant(0.9), λ is the wavelength(λ = 1.5418 A°) (Cu Kα), β is the full width at the half-maximum of the line and θ is the diffraction angle. The grain size estimated using the relative intensity peak for ZrO_2 nanoparticles was found to be 20 nm and increase in sharpness of XRD peaks indicates that particles are in crystalline nature. All different peaks in figure 2 related to ZrO_2 nanoparticles and matched to Joint Committee for Powder Diffraction Studies (JCPDS).

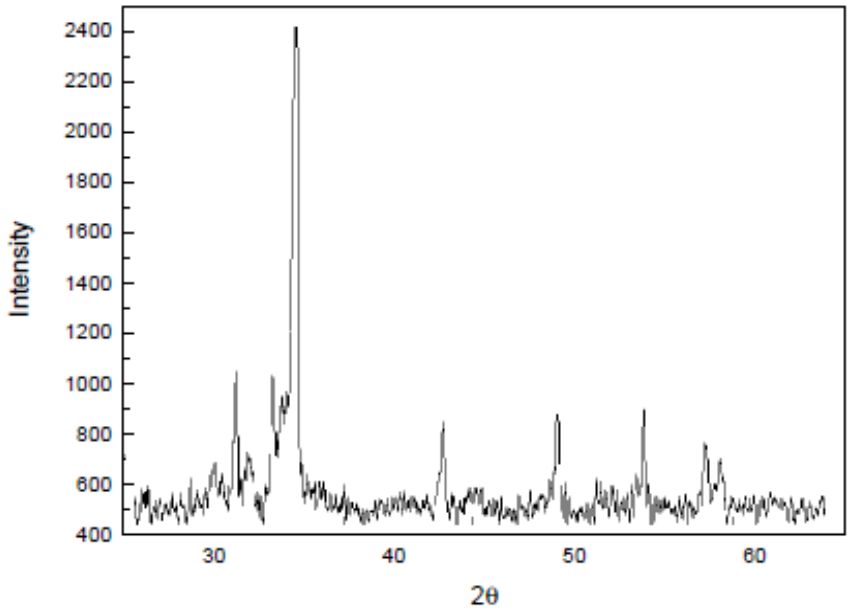

Figure 2. XRD pattern for ZrO_2 nanoparticles

3.2. UV–visible absorption spectra for ZrO_2 nanoparticles

The most dramatic property of nanoparticles is the size evolution of the optical absorption spectra. Hence UV-visible absorption spectroscopy is an efficient technique to monitor the optical properties of quantum-sized particles. The UV–visible absorption spectra of ZrO_2 nanoparticles was shown in Fig. 3; although the wavelength of our spectrometer is limited by the light source, the absorption band of the ZrO_2 nanoparticles have been shows a blue shift due to the quantum confinement in sample compare with bulk ZrO_2 particles. This optical phenomenon indicates that these nanoparticles show quantum size effect [37].

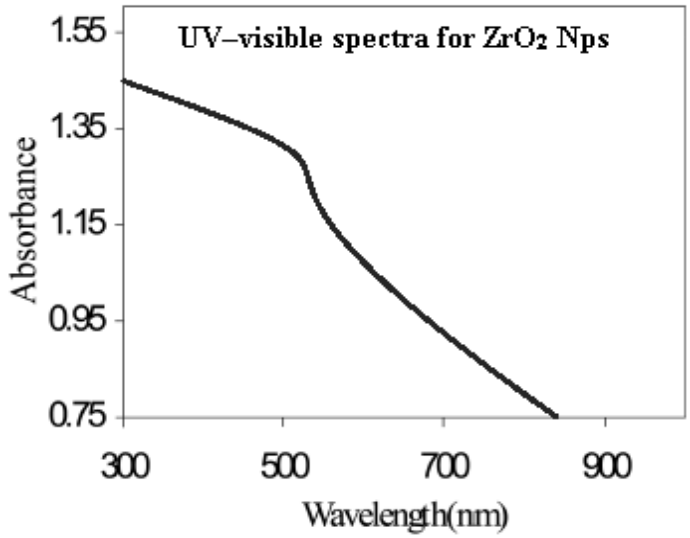

Figure 3. UV-Vis absorption spectra for ZrO_2 nanoparticles.

3.3. Direct electrochemistry of the modified glassy carbon electrode with Cyt c and ZrO_2 nanoparticles

Cyclic voltammetry (CV) was used to characterize the modification of electrodes. The cyclic voltammograms (CVs) of different modified electrodes were obtained in the potential range of 300 – -400 mV in 0.1M PBS (pH 7.0). No redox peak is observed for the CVs of bare GC electrode (figure 4(a)) and ZrO_2 Nps/GC electrode (here not shown). Compared with bare GC electrode, the background current of ZrO_2 Nps-modified electrode is apparently larger, which indicates that the effective electrode surface area is significantly enhanced by use of ZrO_2 Nps to modify the electrode. However, the CVs of Cyt c / ZrO_2 Nps /GC give a pair of well-defined redox and oxidative peaks at −170 and −65 mV at scan rate of 50 mV/s respectively (figure 4(b)), characteristic of heme Fe(III)/Fe(II) redox couples of Cyt c, suggesting that direct electron transfer has been achievede between Cyt c and ZrO_2 Nps-modified electrode. The difference of anodic and cathodic peak potential values were calculated as ΔE = 105 mV. The redox peaks were attributed to the redox reaction of the Cytochrome *c* electroactive center. The formal potential (E^0) for the Cytochrome *c* redox reaction on the Cyt c / ZrO_2Nps /GCE was -117.5 mV versus reference electrode (SCE). The formal potential (E^o) in all electrochemical studies, estimated as the midpoint of reduction and oxidation potentials. ZrO_2 nanoparticles could facilitate fast direct electron transfer between redox proteins and electrode surface. As you know, Cytochrome *c* is the only common heme protein in which the heme is bound to the protein by a covalent linkage. In the cytochrome *c* three-dimensional structure, the hydrophobic aminoacids cluster about the heme on the inside of the molecule and the hydrophilic residues tend to lie on the surface of the molecule. This protein structure could change oxidative iron state (Fe^{3+}) to reductive state (Fe^{2+}) and vice versa by electron transfer. We used of this electron transfer in designing a biosensor for hydrogen peroxide detection.

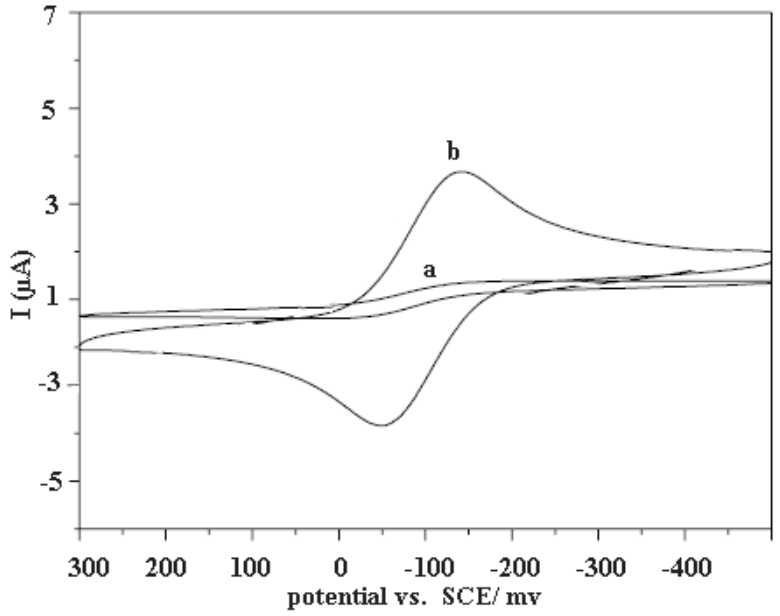

Figure 4. Cyclic voltammograms of (a) bare GCE and (b) Cyt c / ZrO_2Nps /GCE in 0.1 M phosphate buffer (in 0.1 M PBS and scan rate. 50 mV/s).

Fig. 5(a) shows the cyclic voltammograms of the Cyt c / ZrO_2Nps /GCE in 0.1 mol L^{-1} phosphate buffer solution (PBS) of pH 7.0 at different scan rates from 50mVs^{-1} to 400mVs^{-1}. The peak currents increased and the cathodic and anodic peak potentials exhibited a small shift along with the increase of scan rate. At the same time, the cathodic and anodic peak currents increased linearly with the scan rate (not $v^{1/2}$), as shown in Fig. 5(b); in this picture It can be seen that the redox peak currents increased linearly with the scan rate, the correlation coefficient was 0.9861 (ipc = 0.0233v + 2.8533) for catodic peak and 0.9862 (ipa = -.0348v-3.3302) for anodic peak respectively. This phenomenon suggested that the redox process was an adsorption-controlled and the immobilized Cytochrome *c* was stable.

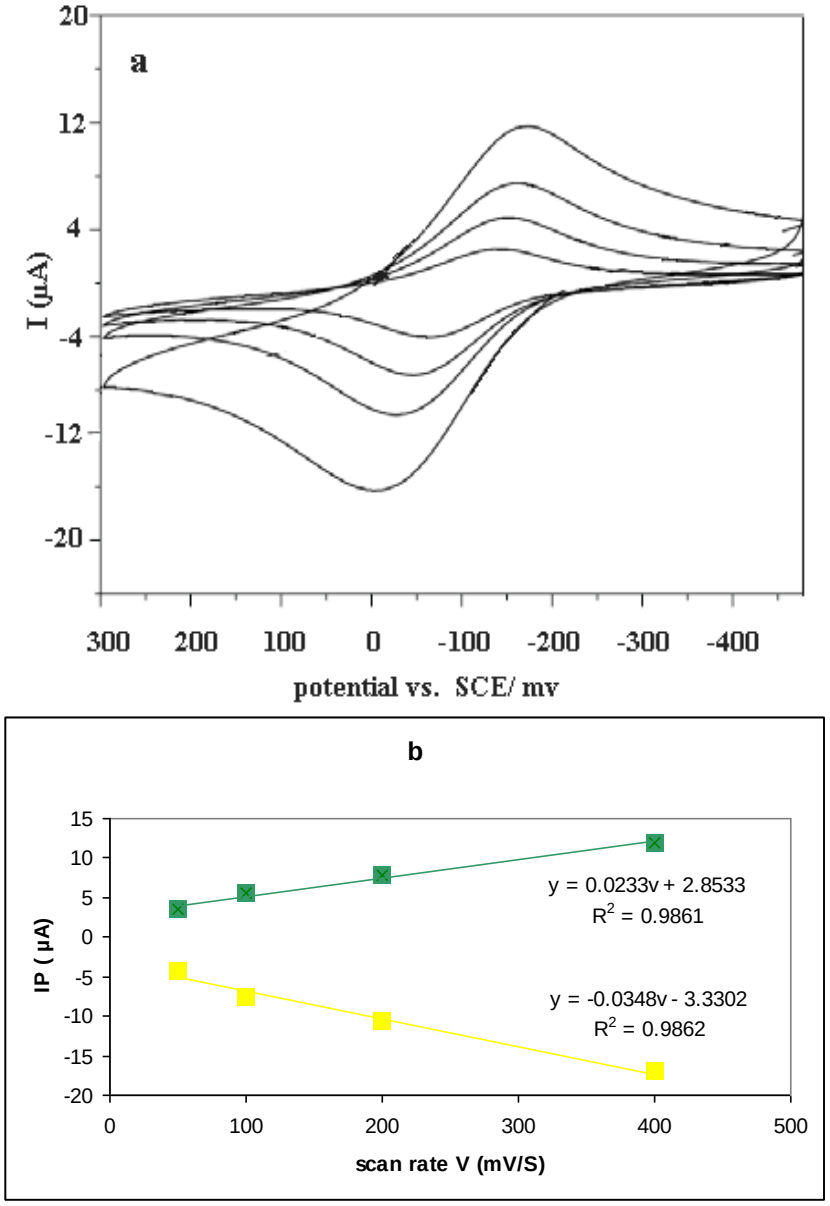

Figure 5. (a) Cyclic voltammograms of Cyt c / ZrO_2Nps /GCE at various scan rates, from inner to outer; 50, 100, 200 and 400 mV s^{-1}, (b) the relationship between the peak currents (ipa, ipc) vs., the sweep rates; Green lines are redox peaks and yellow lines are oxidative peaks.

All these results indicated that the Cyt c immobilized on ZrO_2Nps /GCE surface controlled and quasi-reversible electrochemical reaction process. When the peak-to-peak separation (ΔE) was larger than 200 mV, the apparent heterogeneous electron transfer rate constants (ks) would be easily calculated with the help of Laviron's equations [38] as follows:

$$E_{p,catodic} = E^0 + \frac{RT}{\alpha F} \ln \frac{RTks}{\alpha Fv} \quad (1)$$

$$E_{p,anodic} = E^0 + \frac{RT}{(1-\alpha)F} \ln \frac{RTks}{(1-\alpha)Fv} \quad (2)$$

$$\Delta Ep = Ep_{,anodic} - Ep_{,catodic} = \frac{RT}{\alpha(1-\alpha)F} \quad (3)$$

$$[\log ks = \alpha \log(1-\alpha) + (1-\alpha)\log\alpha - \log\frac{RT}{nFv} - \frac{\alpha(1-\alpha)nF\Delta EP}{2.3\,RT}] \quad (4)$$

Where α is the electron transfer coefficient. Here, n is the number of transferred electrons at the rate of determining reaction. *R*, *T* and *F* are gas, temperature and Faraday constant, respectively (*R* = 8.314 J mol^{-1} K^{-1}, *F* = 96493 C/mol, *T* = 298 K) and ks is the apparent heterogeneous electron transfer rate constants which can be calculated according to ΔEp versus ln *v*. The value of ks was calculated to be 1.83 s^{-1}, which is much higher than that of direct electrochemistry of cytochrome c on electrodeposited nickel oxide nanoparticles (0.23 s^{-1}) [39], or direct electron Transfer of cytochrome c on ZnO nanoparticles modified carbon paste electrode (0.64 s^{-1}) [40]. The ks value directly shows that Cyt c / ZrO_2Nps/GCE complex together enhanced the electron transfer rate between Cyt c and electrode.

3.4. Design a hydrogen peroxide biosensor by use of reduction peaks of Cyt c / ZrO_2/GCE

The electrocatalytic reactivity of Cyt c / ZrO_2Nps/GCE toward H_2O_2 was investigated by cyclic voltammograms. Fig. 6 (a) displays different cyclic voltammograms obtained for the hydrogen peroxide biosensor in PBS (pH 6.0) containing varied concentration of H_2O_2 in the absence of oxygen. The catalytic reduction of hydrogen peroxide at the biosensor can be seen clearly in Fig. 6 (a). With the addition of H_2O_2, the reduction peak current increases obviously while the oxidation peak current decreases (Fig. 6 (a)), indicating a typical electrocatalytic reduction process of H_2O_2. However, no similar cathodic peak corresponding to the reduction of H_2O_2 can be observed at bare GC, ZrO_2Nps /GC electrode under the same condition, so it can be concluded that Cyt c immobilized on ZrO_2Nps/GCE shows good catalytic activity toward hydrogen peroxide. The decreases of oxidative peak happen together with the increases of the reductive peak of Cyt c / ZrO_2Nps/GCE. The electro-catalytic process could be expressed as follows mechanism (equation 4 and 5):

$$\text{Cyt c-Fe (III)} + e^- + H^+ \leftrightarrow \text{Cyt c-Fe (II) } H^+ \quad \text{at the electrode surface} \quad (4)$$

H_2O_2 + Cyt c-Fe (II) $H^+ \rightarrow$ Cyt c-Fe (III) + H^+ + H_2O_2 in the solution (5)

The calibration curve (Figure 6b) shows the linear dependence of the cathodic peak current on the H_2O_2 concentration in the range of 40 to 270 μM. In Figure 6(b) at higher concentration of H_2O_2, the cathodic peak current decreased and remains constant. As can be observed, the sensor response shows good linearity in this range. The correlation factor, R^2 was found to be 0.9996. Since the H_2O_2 released is in relatively small micro-molar range, sensor response at such low concentrations assumes great significance. This implies electrocatalytic property of electrode. Hydrogen peroxide detection in micro-molar range is of importance in plant&animal physiology [41-42]. In general, H_2O_2 is released as a stress response to structural damage in plant tissues [43] and also it level involved in a number of biological events and intracellular pathways that have been linked to several diseases [44-45].

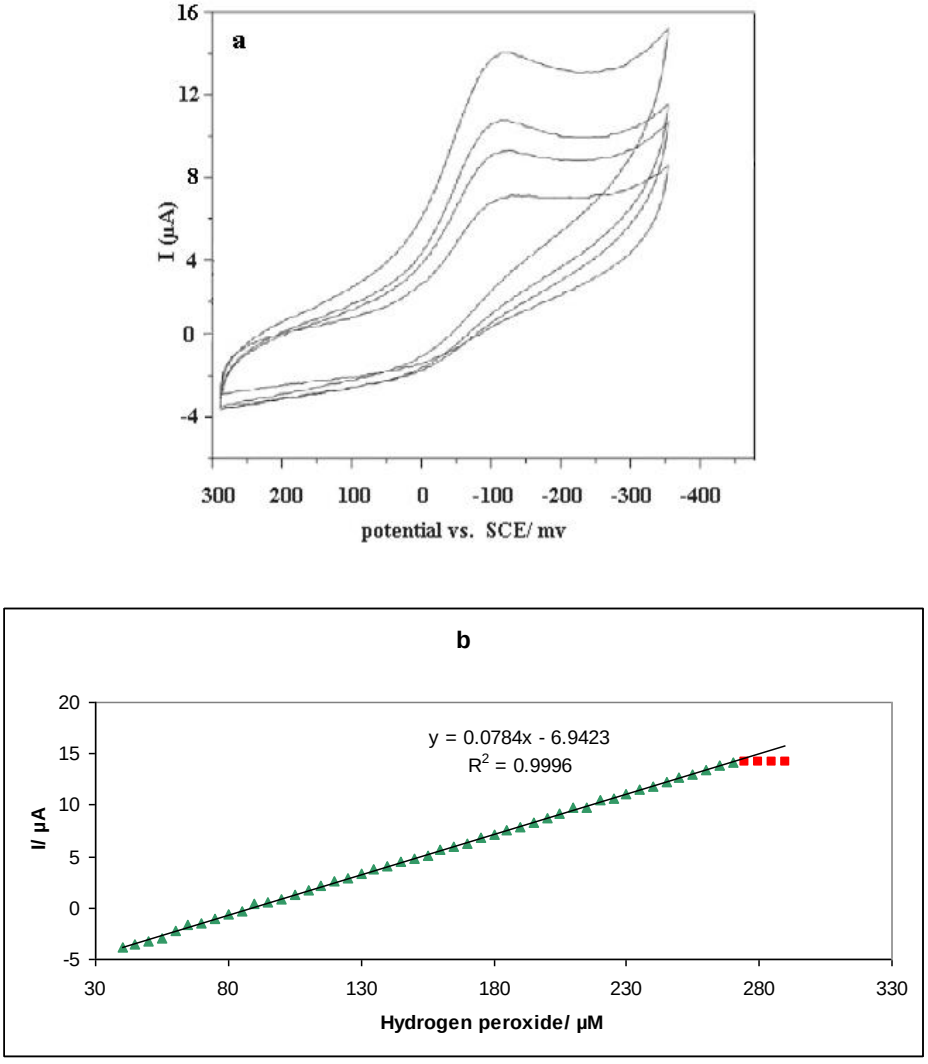

Figure 6. (a) Cyclic voltammograms obtained for Cyt c / ZrO_2Nps/GCE in 0.1M phosphate buffer solution (pH 7.0) for different concentrations of hydrogen peroxide and (b) the relationship between cathodic peak current of Cyt c and different concentrations of hydrogen peroxide (scan rate: 100 mVs^{-1}).

3.5. Influence of pH on biosensor response

In order to obtain an efficient biosensor for H_2O_2, the influence of pH and applied potential on the response of Cyt c / ZrO_2Nps/GCE electrode were investigated. The change of chronoamperometric current with the pH under constant hydrogen peroxide concentration (10 mM) is shown in Fig. 7. As can be seen, the maximum response appears at pH 7.0. So the used of PBS with pH 7.0 was good select for our experiments. In this section pH 7.0 was the best for biosensor response

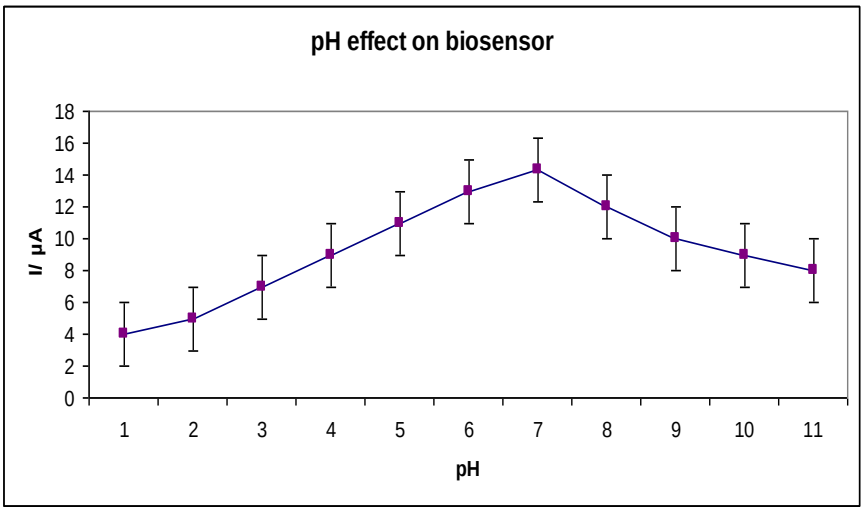

Figure 7. Typical diagram of response biosensor to pH changes; the maximum of response observed at pH.7.

3.6. Efficiency of designed H_2O_2 biosensor

The stability of Cyt c / ZrO_2/GCE towards the catalytic reduction of H_2O_2 has been studied. The relative standard deviation is 6.0% for 10 successive determinations of 40µM H_2O_2. When the modified electrode is stored at 4 °C and measured intermittently (every 2 or 3 days), after 30 days, the biosensor can retain 91% of its initial response, indicating the good stability of this biosensor. The reproducibility of 3 proposed biosensors prepared with the same method was estimated, by the response, to 40µM H_2O_2. The result reveals that the biosensor has satisfied reproducibility with a relative standard deviation of 7.3%, which showed the modified electrode had a good repeatability. Interface materials had not high effect on operation of this biosensor. In total, the mentioned hydrogen peroxide biosensor based on Cyt c / ZrO_2/GCE showed a fast response, high sensitivity, good reproducibility and stability.

4. CONCLUSION

Designing novel biosensors with use of nanotechnology considered for many researchers in recent years. In this research we used of ZrO_2 as facile electron transfer between cytochrome c and

glassy carbon electrode. Our results led to design a new biosensor for hydrogen peroxide detection. The preparation process of the proposed biosensor was convenient, and the resulting showed that mentioned biosensor had high sensitivity, low detection limit, and good stability and also interface materials had not high effect on operation of it.

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