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Electrochemical Biosensor Hemoglobin Immobilization Determination of the Breast Cancer Drug (Adriamycin)

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ABSTRACT

Electrochemical biosensor is an effective tool for pharmaceutical analysis due to its simplicity, specificity, sensitivity, fast, cost effective and repetitive measurements with miniaturized and portable devices. The paper illustrates the detail methodology for development of an electrochemical biosensor based on hemoglobin (Hb) film modified for anticancer drug adriamycin (ADM). Square wave voltammetry (SWV) studies of the electrodes before and after immobilizing of HB shows the successful formation of a selectivity of the electrode. The proposed Hb based biosensor allows quantitation over the range 1 to 80×10^{-8}M, The suggested biosensor method can be successfully applied to the detection and determination of anticancer drug adriamycin in different drug formulations and can be suggested as a suitable sensor for analysis of Adriamycin in biological samples.

Keywords: Electrochemical biosensor, Adriamycin, Hemoglobin, Breast cancer.

تقدير دواء سرطان الثدي (الادريامايسين) بواسطة تركيز الهيموغلوبين من خلال تحضير المتحسس الحيوي الكهروكيميائي

الملخص

أن المتحسسات الحيوية الكهروكيميائية وسيلة فاعلة في تقدير المستحضرات الـصيدلانية بـسبب سـهولة تطبيقاتهـا، وحساسيتها العالية وتكلفتها الاقتصادية، في هذا العمل تم تطوير طريقة لتقدير احد ادوية العلاج الكيميائي لمرضـى سـرطان الثدي (الادريامايسين) باستخدام تقنية فولتامتري الموجة المربعة ومن خلال تحوير القطب الصلب مع الهيموغلوبين حيث يمكن تقدير كميات من الادريامايسين ضمن المدى ($1_80\times10^{-8}$M)، بدوره يعمل الهيموغلوبين على تحـسين مواصـفات القطـب ويجعل امكانية تطبيقه على النماذج الحيوية ممكناً.

الكلمات الدالة: المتحسسات الحيوية، الادريامايسين، الهيموغلوبين، سرطان الثدي.

INTRODUCTION

Cancer is a very serious health problem in all developed countries. Since the beginning of the 21st century, cancer has becomes one of the most common primary causes of death, of which malignant breast tumors predominate for women (Risbridger *et al*., 2010; Cosimo and Baselga, 2010 ; Lin *et al*., 2010) and prostate cancer in men (Capitanio and Suardi, 2011; Lawrentschuk and Klotz, 2011; Oon *et al*., 2011). Treatment options are based on surgical removal of cancerous tissue, radiotherapy and chemotherapy. Chemotherapy approaches interfere with quite different cellular and molecular processes. Intercalators belong to the important group of drugs that are used in

cancer therapy. Biosensor offers a very promising tool with its application in the field of pharmaceutical, biomedical and environmental compounds. For the last two decades, conducting polymers have emerged as one of the most interesting materials for the fabrication of biosensors and electrochemical sensors (Mulchandani and Wang, 1996).

Adriamycin

Adriamycin(ADM)(7S,9S)-7-[(2R,4S,5S,6S)-4-amino5-hydroxy-6-methyloxan-2-yl]oxy-6,9,11-trihydroxy-9-(2hydroxyacetyl)-4-methoxy-8,10-dihydro-7H-tetracene-5,12dione. Fig. (1) is an anticancer (antineoplastic) chemotherapy drug classified as an anthracycline antiobiotic, commercialized in the form of chloride salt and sold as Adriamycin (Blum and Carter, 1974). Wide spectrum of chemotherapeutic applications and anti-neoplasic action widely used as an antitumor agent, and exhibits antitumor activity against solid tumors, such as breast and lung cancers (Ghirmai *et al*., 2005) is used in the treatment of various forms of sarcoma and cancer, including bladder cancer, breast cancer, leukemia, liver cancer, head and neck cancer, and lung cancer (Reza and Zahra, 2016).Its strong DNA binding properties lead the discoverers of the antibiotics to suggest that the drugs receptor site is DNA (Marco *et al*., 1964 ; Arcamone *et al*., 1969). Most of the available evidence indicates that it binds to double helix mainly at CG-GC steps, the amino sugar being determinant for intercalation to occur (Berg *et al.*, 1981).

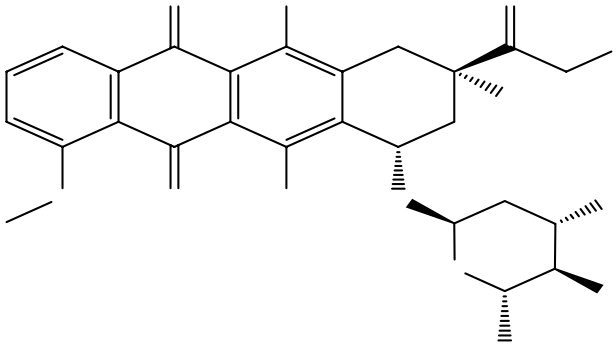

Fig. 1: Moleculare structure of adriamycin

Biological Effect of Adriamycin

Kiyomia *et al*. performed some experiments to identify the intracellular specificity of the differential cytotoxic effects of adriamycin on neoplastic and normal cells. These results suggest that adriamycin exerts cytostatic effects on neoplastic and normal undifferentiated cells through the inhibition of DNA synthesis by DNA intercalation, and cytotoxic effects on neoplastic cells through the accumulation of reactive oxygen species resulting from low scavenger enzyme activities. The cytotoxic effects on normal differentiated cells may be related to the high levels of production reactive oxygen species due to high mitochondrial NADH-cytochrome C reductase activity (Kiyomiya *et al*., 2001). Adriamycin is commonly used to treat some leukaemias, Hodgkin's and non- Hodgkin's lymphomas, as well as cancers of the bladder, breast, stomach, lung, ovaries, thyroid, soft tissue and bone sarcomas, multiple myeloma, among others.

Reactive Oxygen Species Generation by Adriamycin and its Cardiotoxicity

Generating reactive oxygen species causing lipoperoxidation that cause damage cell membranes, apoptotic changes via interaction with iron ions and activation of NFκB belongs to the other important effect of adriamycin *in vivo* (Minotti *et al*., 2004; Keizer *et al*., 1990). The scheme of the effects of the generated radicals is shown in Fig.(2). Anthracyclines including adriamycin have long been said to induce cardiotoxicity by mechanisms other than those mediating their antitumor effectiveness, a concept which has raised hopes to design strategies for protecting the heart while not diminishing tumour response. Here we describe the most recent advances that may

help to shed light into the mechanisms by which anthracyclines induce cardiotoxicity in experimental or clinical settings (Minotti *et al*., 2004; Singal and Iliskovic, 1998). To reduce their cardiotoxicity, nanoparticles based approaches for encapsulation of these drugs seems to be very convenient for this purpose (Patil *et al*., 2008).

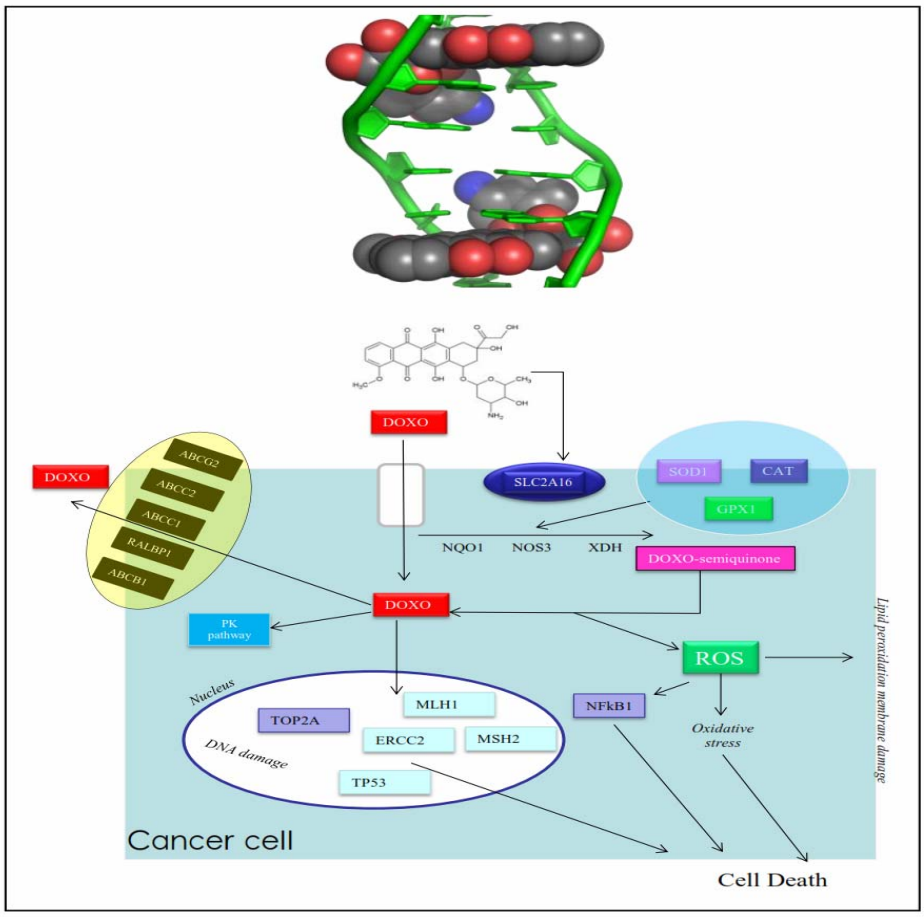

Fig. 2: Scheme of adriamycin interactions with different proteins in a cancer cell

Adriamycin and Techniques Used

The pharmacological effects of this drug have been ascribed to the interaction with double helix of DNA and the anthracycline moiety which causes inhibition of replication and transcription of DNA in cancer cells (Lown, 1993). However, clinical use of ADM is still limited for its cardiotoxicity that is usually caused by oxidative stress. Like all anthracyclines, ADM primarily works by intercalating DNA, but the mechanism of toxicity is also based on cell damage induced by reactive oxygen species, a mechanism in which free iron plays an important role. This opens a new field for the application of antioxidants (free radical scavengers and/or iron-chelating agents) in the potential protection of heart and other organs from ADM toxicity without impairing its antitumour efficacy (Injac and Strukelj, 2008). So, it is unequivocal that the development of sensitive and reliable analytical tools for the determination of ADM is a basic requirement for the study of this analyte in different types of samples with complex matrices. For this purpose, different analytical techniques have been used, and the most common among them is high performance liquid chromatography coupled with different detectors such as UV-Vis (Chin *et al*., 2002), tandem MS (Arnold *et al*., 2004; DiFrancesco *et al*., 2007), chemiluminescence (Ahmed *et al*., 2009), fluorimetric (DiFrancesco *et al*., 2007), and electroanalytical detectors (Ahmed *et al*., 2009). The capillary electrophoresis coupled with laser induced fluorescent detector (Anderson *et al*., 2003) is noted as another powerful hyphenated measurement technique for the separation and trace level determination of ADM and its intermediates from complex biological matrices. Besides, UV-Vis

spectrophotometry (Siméon *et al*., 1999), fluorimetry (Liao *et al*., 2005), Raman spectroscopy (Liu and Danielsson, 2007), immuno assay analysis (Karukstis *et al*., 1998), and electroanalytical measurement techniques (Jemelkov *et al*., 2009 ; Hillard *et al*., 2008), have also been used for the determination of ADM in different samples. Electroanalytical methods represent an appropriate alternative to the above mentioned techniques, because of low cost of instrumentation, fast and sensitive performance of analysis, and simpler sample pretreatment procedures. Voltammetric methods, based on the use of different working electrodes, have proven to be convenient for the determination of this drug, thanks to the fact that its structure contains electroactive groups (Vacek *et al*., 2009). The determination of ADM is usually carried out using mercury-based electrodes (Forster, 1996) as well as different types of carbon based electrodes (Komorsky-Lovric, 2006). It is well known that ADM is strongly adsorbed in different electrode materials such as mercury (Yan *et al*., 1997), carbon paste, different types of graphite (Komorsky-Lovric, 2006), and glassy carbon (Jemelkov *et al*., 2009). This property has been utilized for electroanalytical determinations of ADM at trace levels (Baldwin *et al*., 1981). Besides, ADM was monitored by different electrodes in complex biological matrices, e.g. to assess its efflux from monolayers of cancer cells (Vacek *et al*., 2009), and later, from single isolated cells of the same cell lines (Oliveira-Brett *et al*., 2002), and in a case study on assessing transport at live cell preparations (Yi and Gratzl, 1998). Finally, electrochemical methods have been used in the exploratory investigation concerning the interaction of ADM and DNA (Nair and Gratzl, 2004).

Hemoglobin (Hb) is a tetrameric protein that consists of four polypeptides and each polypeptide chain contains a heme group that serves as the active center (Kafi *et al*., 2007; Sun *et al*., 2004). It has been demonstrated that on conventional solid electrodes, the fast electron transfer between Hb and the electrode is not possible because of its deep entombing of the electroactive group in the structure of Hb (Gu *et al*., 2001).

Cancer is one of the most deadly diseases, and current detection options are ineffective. Recently, a large amount of research has been conducted for the development of biosensors which are able to detect cancer biomarkers. Many biosensors have been created for cancer detecting purposes. We examined literature reviews onlining current biosensing methods. These reviews provided an overview of the sensing techniques that are currently in existence as well as evaluation of their effectiveness. These paper help to showcase the feasibility and effectiveness that a biosensor has detecting cancer. In this paper I discuss the signal transduction systems, Electrochemical sensor, to use in cancer biosensors. Since cancer is such a deadly disease, an accurate biosensor is needed that can detect cancer earlier than current methods. In this paper, we will provide an overview of each method along with an evaluation to determine which cancer detecting system is the best.

EXPERIMENTAL SECTION

Instrumentation

Voltammetric measurements were carried out on 797 VA Computrace (Metrohm, Switzerland).

A conventional three electrode systems comprised were carried out using three electrodes cells, including Reference electrode (RE) as Ag/AgCl 3 M KCl (metrohm, AG, 60728.020), Pt electrode was used as Auxiliray electrode (metrohm, AG, 6.0343,000). The (3.0 mm) bare Graphite electrode (Home made, Germany) as working electrode , graphite rod electrode was fabricated by sealing commercial graphite rods (diameter of 3.05 mm; SGL Carbon, Bonn, Germany, www.sglgroup.com) in glass tubes (outer diameter 5 mm) using epoxy glue (UHU plus endfest 300). All experiments were performed at room temperature (25.0±0.5˚C). The pH measurements were realized using a digital pH meter (HANNA, Portugal). For cleaning the working electrode ultrasonic cleaner was used (model; CD-4820, China).

Chemicals

The ADM and its pharmaceutical dosage form were supplied from Koçak Farma Inc. (Istanbul, Turkey). Haemoglobin Bovine (molecular weight: 64,500) was supplied from HiMedia Laboratories Pvt. Ltd.(Mumbai, India). Phosphate, Tris-HCl and Britton Robinson buffers were used as supporting electrolytes. All solutions were protected from light and used within 24 h to avoid decomposition. chemicals were used of analytical reagent grade (Fluka, BDH) used as received without any further purification, and doubly distilled water was used throughout the experimental work.

Cleaning and Polishing Electrode

The (3.0 mm) Graphite electrodes (Home made, Germany), which were constrained in a glass rode, were polished with emery paper (P400/P800/P2000) and aluminaoxide in different (3.0 µm, 0.3 µm, 0.1 µm, 0.05 µm) repeatedly with afterwards thoroughly rinsed with double distilled water (we should be take care to rinsed with double distilled water after using every size of Alumina polish set) then sonicated for (15 min) in double distilled water and allowed to air dry before further modification

Preparation of the Hb Electrode

Electropolymerisation of Hb at the surface of bare GE was carried out by using cyclic voltammetric method in aqueous solution containing 1.0×10^{-3}M Hb in 0.2 M PBS of pH 7.0. The HB molecules are reduced to free radicals and rapidly combine with the GE surface.After 8 cycles, the peak currents remain constant; therefore, 8 cycles are chosen as a representative for the modification process. A uniform film is produced on GE surface, which indicates that the HB has been deposited on the GE surface by electropolymeriztion method.

RESULTS AND DISCUTION

Voltammetric Behavior of ADM

Voltammetric measurements were carried out in the presence of 0.2 M PBS at pH (4.0-10.0) by square-wave voltammetry (SWV). In the case of model solutions of ADM, The analyze was added with a micropipette to the supporting electrolyte consisting 0.2 M PBS (10.0 mL). the potential was swept in the range from -1000 *mV* to -200 *mV vs.* Ag/AgCl, the peak potential at (-601) *mV* under default conditions of the instrument (Fig 2) shows the square-wave voltammogram obtained for at pH 7 of a bare graphite electrode

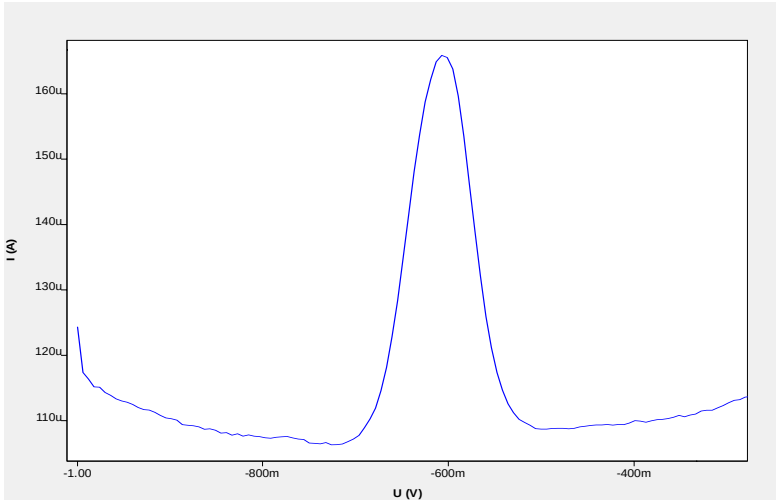

Fig. 2: square-wave voltammetry of ADM in 0.2 M PBS at pH 7.0

Optimization of parameters for ADM

To optimize the instrument parameter conditions for measurements by quare-wave voltammetry (SWV) ttechnique, the quare-wave voltammogram of (50.0) nM of ADM in phosphate buffer (pH 7.0) was studied previously and was used as supporting electrolyte. The table below show the values of all parameters that give the best peak of ADM and these conditions were used for all measurements.

Table 1: Influence the variant conditions for (50.0) nM ADM in PBS(7.0) to give optimum peak resolution with higher current

Optimum Condition	Values
Start Potential (V)	-1.0
End Potential (V)	-0.2
Deposition potential (V)	-0.3
Deposition time (s)	5
Equilibration time (s)	5
Voltage step (V)	0.0065
Amplitude (V)	0.05
Frequency (Hz)	60

Influence of pH and the structure of ADM

In view of the fact that the ADM molecule contains a quinone functional group which is electroactive on Graphite electrode, a detailed study of the behavior of ADM at GE in the potential span from -1000 to -200mV in the 0.2 M phosphate buffer (pH 4.0-10.0) was performed by square-wave voltammetry. Based on the shape and intensity of the reduction signal of ADM, the pH 7.0 was selected as an optimal value for the determination by SWV, while at pH 8.0 to 10.0 a significant decrease of the peak current is observed. The obtained peak shifts to the more negative potentials with the increase in the pH, and the plot of Ep versus pH is linear as shown in (Fig. 4), with the equation of best fit being: Ep= -0.328-0.038 pH (r = 0.9903). All this indicates a complex electrode process in which electron exchange is significantly influenced by protons. At pH 10.0, the color of ADM solution becomes violet due to the conjugation in the quinone functional group.

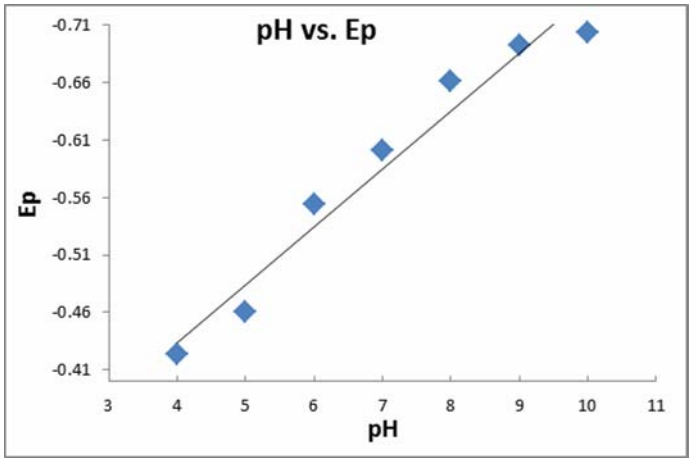

Fig. 4: The influence of the pH on the ADM signal obtained with GE (50.10 ng mL^{-1})by SWV and on the peak potential.

Reduction mechanism of ADM on graphite electrode

The previous chronocoulometric measurements showed that the number of electrons involved in the reduction of another quinone molecule sodium 1,4-dihydroxy-9, 10-anthraquinone-2 sulphonate at the graphite electrode is two (Guin *et al*., 2010). Additionally, the extensively studied reaction of para-benzoquinone, having a similar electroactive center, showed that its reduction proceeds in two electron-transfer steps coupled with proton acceptance (He *et al*., 1990). Hence it seems plausible to think that the same process takes also place on the GE at pH 7.0, and the overall reaction can be described as follows Fig. (5).

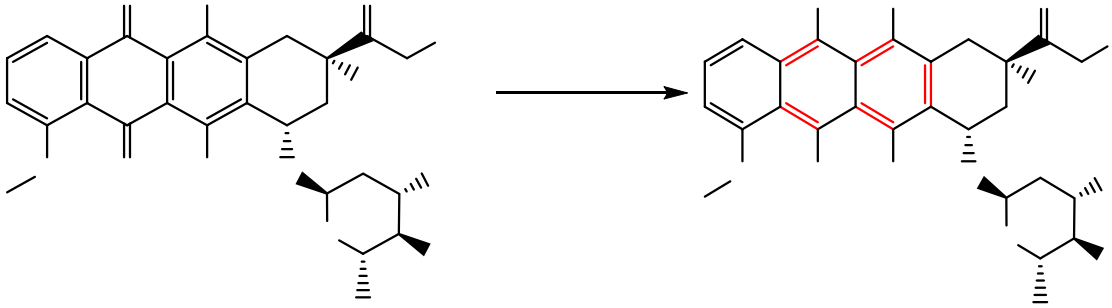

Fig. 5: Reduction mechanism of ADM on GE at pH 7.0.

Effect of the concentration (calibration graph of ADM) using SWV

The calibration curve of ADM was constructed using the optimum conditions in (Table 1). The SW. Voltammograms were recorded for serial additions of (10^{-7})M ADM in P.B.S. (pH 7.0) at (25^0C) using GE. The peak current plotted against the ADM concentration is shown in Fig. (6).

It is very clear that the intended relation shows a straight line, the straight line between the concentration range (5×10^{-7}- 75×10^{-7}) M and Ip which gave a correlation coefficient (R^2= 0.9756)

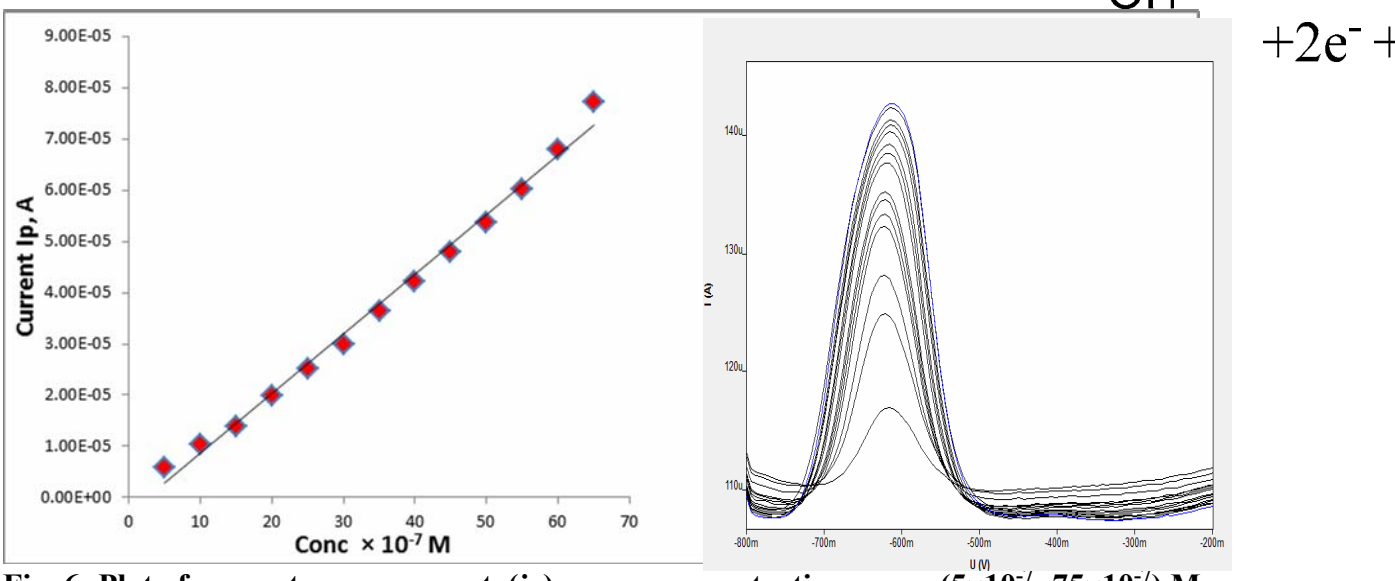

Fig. 6: Plot of current measurement (*ip*) versus concentration range (5×10^{-7}- 75×10^{-7}) M of ADM

Direct Electrochemistry of Hb

In this paper, a film Hb was fixed on the surface of the GE with electrostatic growth mode. Hb (isoelectric points at 7.0) has considerable negative surface charges at pH 7.0. Hb showed good direct electron transfer behavior on the GE and the direct electrochemistry of Hb on the modified electrode was studied by cyclic voltammetry.

Fig. (7), showed cyclic voltammograms of modified electrode in pH 7.0 phosphate buffer solution. A pair of well defined, quasi-reversible cyclic voltammetric peaks (curve a)was found (Ep_a= -0.205V) and (Ep_c=-0.251 V) at the scan rate 0.05 Vs^{-1}. The peaks were located at the potential characteristics of the heme Fe(III)/Fe(II) redox couples of proteins. The results indicated that all of the electroactive Hb Fe(III) in the film was reduced to Hb Fe(II) on the forward scan and then reoxidized to Hb Fe(III) (Wei Sun *et al*., 2007).

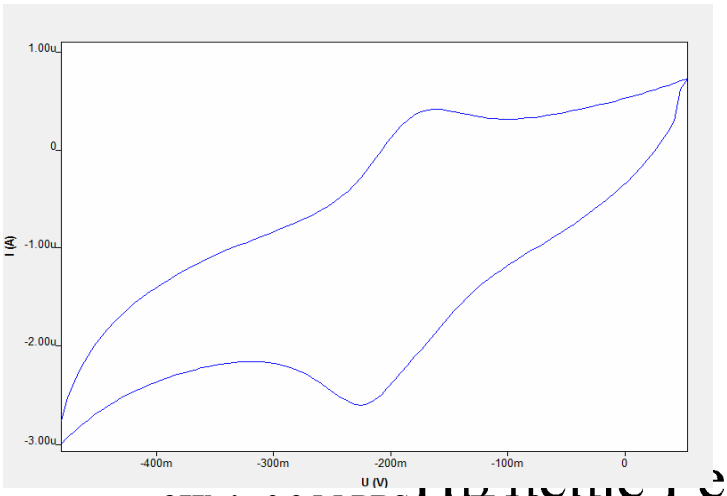

Fig. 7: Cyclic voltammetry of Hb in 0.2 M PBS at pH 7.0 with a scan rate of 50 mV s^{-1}

Electropolymeriztion of HB onto GE

Electropolymeriztion is a facile and efficient approach to immobilize an organic film onto solid state electrode surface because film properties, such as thickness, permeation and charge transportation, can be adjusted by controlling electrochemical parameters. Fig. (8) shows the successive CV curves during the electropolymeriztion of HB from a phosphate buffer solution (pH 7.0) containing 1.0 × 10^{-3}M HB. The HB molecules are reduced to free radicals and rapidly combine with the GE surface. After 8 cycles, the peak currents remain constant; therefore, 8 cycles are chosen as a representative for the modification process. A uniform film is produced on GE surface, which indicates that the HB has been deposited on the GE surface by electropolymeriztion method.

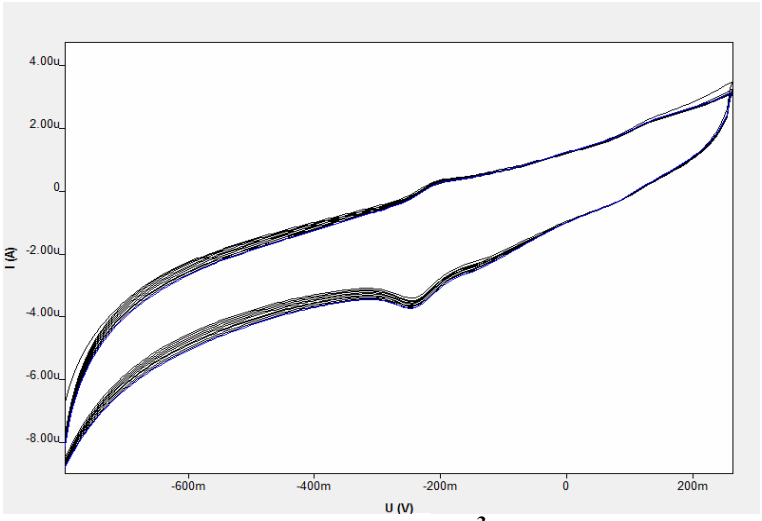

Fig. 8: Electropolymerization curves of 1.0 × 10^{-3}M Hb in 10ml pH 7.0 PBS at GE
Inset: the influence of the cycle and scan rate on the oxidation peak current

ADM determination of the Hb electrochemical biosensor

The SWV technique was used to investigate the electrochemical behavior of the G bare and G modified electrodes in the attend ADM with the SWV sweeping from -1000 mV to -200 mV versus Ag/AgCl reference electrode. Fig. (9) shows the electrochemical SWVs taken for 3.0 $\times 10^{-8}$ M to 3.0$\times 10^{-7}$ M ADM on the surface PHb/GE. The current intensity as Fig. (9) shows the relationship between oxidation peak current and the ADM concentrations.

Hb in electron transfer boosting of the sensing layer can be attributed to its good anchoring to ADM, good electron mediatory, good electron pathway and favorable sensitivity and selectivity due to resulting Hb/GE showed good electrocatalytic activity towards ADM reduction. Fig. (6) shows the SW. voltammograms obtained at the GE in the presence of different amounts of ADM. When ADM was added into pH 7.0 buffer solution, the peak currents increased and enhanced due to the immobilized Hb film in the GE, which provided a fast direct electron transfer reaction between the heme of Hb and the electrode surface, heme can react with ADM to form a first intermediate of compound I, which has catalytic activity to ADM. The catalytic procedures can be explained as follows:

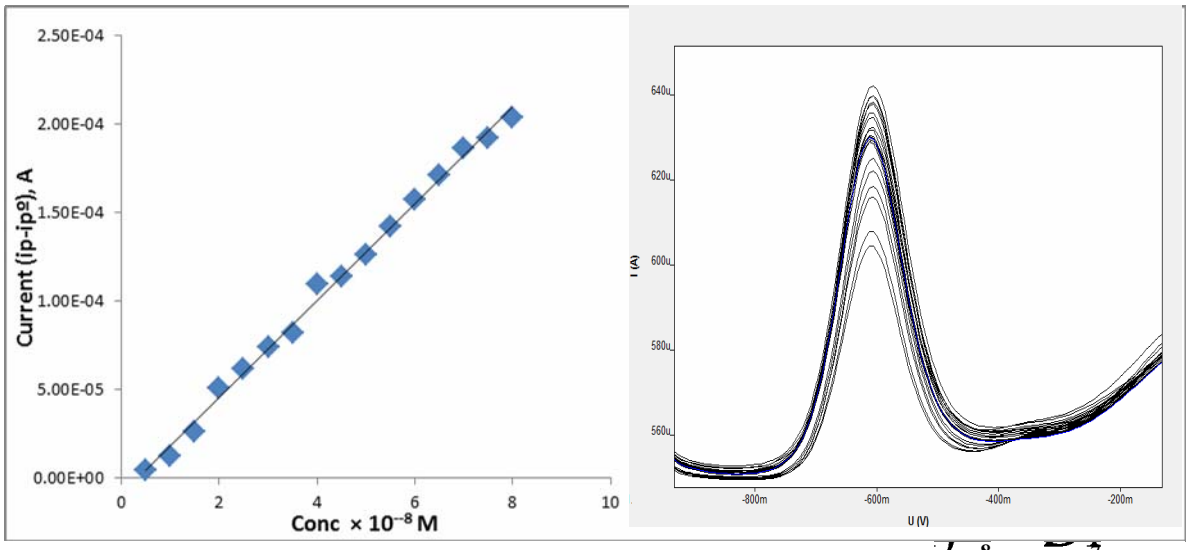

Fig. 9: Plot of current measurement (*ip*) versus concentration range (3× 10^{-8}- 3× 10^{-7}) M of ADM on pHb/GE

$$\text{Compound I} + \text{ADM} \rightleftharpoons \text{HbFe(III)} + e^{-}$$

$$\text{HbFe(II)} + O_2 \rightleftharpoons \text{HbFe(II)}\text{-}O_2 + 2e^{-} + 2H^{+}$$

CONCLUSION

In this work, an electrochemical sensor was fabricated based on modification by the direct immobilization and electron transfer of hemoglobin which were achieved on graphite electrode for determination of Adriamycin as a chemotherapy drug. Direct immobilization and electron transfer of hemoglobin on GE can be established as a foundation for constructing the new generation of biosensors without using supporting films and redox mediations. Hemoglobin structure of exhibited a suitable electroactive substrate for oxidation/reduction of Adriamycin. The electrochemical parameters including: pH, kind of buffer (Phosphate, Tris-HCl and Britton Robinson buffers were used as supporting electrolytes), parameters have been optimized. Under the optimum conditions,

there was a good linear relationship between cathodic peak current and concentration of Adriamycin . The proposed method displayed excellent characteristics as simplicity, economy, good sensitivity, selectivity, reproducibility and rapid response, so it is suggested as a suitable sensor for the analysis of Adriamycin in biological samples.

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