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Investigation of Electrochemical Behavior of Bilirubin at Unmodified Carbon Paste Electrode

Maedeh Akhoundian,[1] Taher Alizadeh[1,*] and Guoqing Pan[2]

[1]*Department of Analytical Chemistry, Faculty of Chemistry, University College of Science, University of Tehran, P.O. Box 14155-6455, Tehran, Iran*
[2]*Institute for Advanced Materials, School of Material Science and Engineering, Jiangsu University, Zhenjiang 212013, China*

*Corresponding Author, Tel.: +982161112788; Fax: +982161112788
E-Mail: talizadeh@ut.ac.ir

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Abstract- Bilirubin is predominantly formed in the liver as a result of breakdown of hemoglobin. Knowing the concentration of bilirubin in serum is important in evaluating the health of the liver, and for the diagnosis of hyperbilirubinemia (a condition that afflicts approximately 60% of full term and 80% of pre-term newborns). In this work, a carbon paste electrode (CPE) has been used to study the electrochemical oxidation of bilirubin (BR) and its feasibility to be performed at unmodified or untreated carbon paste in an effort to propose a simple voltammetric method for detection of bilirubin. Irreversible oxidation of BR at CPE had occurred at about +0.32 V *vs*. Ag/AgCl as a diffusion-controlled process, when suitable pH for this redox transformation was either a mild alkaline solution (of Phosphate buffer, pH 8) or a neutral supporting medium. After having chosen the DPV mode and optimizing the electroanalytical parameters, the oxidation responses of the analyte has been justly proportional to concentration in the range of 3.5–25 µmol. L^{-1} with LOD of 1.2 µmol. L^{-1} in phosphate buffer.

Keywords- Bilirubin, Electrochemical behavior, Carbon paste electrode, Differential pulse voltammetry, Biosensing

1. INTRODUCTION

Bilirubin is a yellow colored pigment, produced as a waste material by the catabolism of heme, which is excreted through bile, urine and stool. BR exists in the blood as direct and indirect [1]. Indirect or unconjugated BR is the albumin-bound, water soluble form which circulates in the plasma. The indirect BR is then transported to the liver where it is conjugated with glucuronic acid to form direct or conjugated BR and excreted [2]. Besides these forms, albumin unbound, toxic, free bilirubin can also be present in the blood. Studies show that the bilirubin toxicity is better correlated with free bilirubin than total bilirubin concentration. Unconjugated hyperbilirubinemia, a common medical condition in newborns is characterized by the presence of free bilirubin in the blood which can cause serious health problems for the infants. Unbound, unconjugated serum bilirubin has been an elusive target for facile quantitative assays. Its concentrations in newborn infants are much lower than that of total serum bilirubin. However, it is the level of unbound bilirubin that controls the rate at which bilirubin can escape the blood compartment and presents danger to the infant because of its neurotoxic effects [3]. Thus, monitoring of the level of unbound BR is so important from medicinal point of view. Determination of BR can be carried out by means of colorimetric [4,5] and fluorimetric [6-12] methods as well as electrochemical [13-21].

Utilizing the carbon paste electrodes (CPE) is one of the easiest and cheapest as well as precise way in electrochemical determinations. Carbon paste electrodes have been thoroughly used since Adams in 1958 described the first electrode [22]. They present the advantages of being cheap, easy to prepare, chemical inertness, showing a wide potential window and low background currents and facile to modified with different organic, inorganic, biological or natural products. When the bulk of the electrode material is modified, the surface of the modified carbon paste electrode can be easily renewed and repetitive measurement can be achieved [23,24]. Renewing the surface of CPE avoids the memory effects and the contamination or deactivation of the surface between consecutive measurements. It therefore provides accurate measurements in pharmaceutical and biological samples [25-29]. Such electrodes are based on the dispersion of graphite particles with a water-immiscible, non-conducting pasting liquid.

There are few reports of the use of carbon materials in fabrication of bilirubin sensors [30-33]. But concerning unmodified carbon paste and its use to analyze the BR containing samples, there is, to our knowledge, no report at all despite of its notable ease, accessibility and cheapness. These findings had propelled our decision to examine carbon paste-based electrodes in an investigation on the electrochemistry of bilirubin.

BR is liposoluble at physiological conditions but poorly soluble in laboratory aqueous solvents. Therefore, the electrochemistry of BR has been investigated in detergents [34] or in organic solvents such as dimethyl sulfoxide (DMSO) and dimethyl formaldehyde [35,36]. Even in the works in which the aqueous solution was applied [37,38], the BR stock solution

was prepared by dissolving it in a basic solvent and the lower BR concentrations were subsequently obtained by dilution in aqueous buffers. In the present study also, DMSO was used to prepare the bilirubin stock solution and the dilution was performed using phosphate buffer.

In this work, the electrochemical oxidation of bilirubin at unmodified CPE was studied, using cyclic and differential pulse voltammetry techniques in phosphate buffer.

2. EXPERIMENTAL

2.1. Apparatus and chemicals

Electrochemical data were obtained with a three-electrode system using a potentiostat/galvanostat IVIUM Vertex model. The unmodified carbon paste sensor was used as the working electrode. A platinum wire and an Ag/AgCl electrode were used as the counter and reference electrodes respectively. Bilirubin and all other chemicals were of analytical grade and purchased from Merck (Darmstadt, Germany).

2.2. Preparation of carbon paste electrode (BR sensor)

The unmodified carbon paste (work) electrode was prepared by thoroughly hand mixing of 75 mg of graphite and 25 mg eicosane in a mortar. The resulting paste was then packed firmly in the electrode hole. The working carbon paste electrode has a surface diameter of 4 mm and it comprises a hollow teflon tube in which a stainless steel rod is inserted acting as the electric contact between the carbon paste and the potentiostat. The surface of the electrode was polished then on a soft sheet of paper. The CPE surface was renewed by extruding ca. 0.5 mm of carbon paste off with subsequent smoothing with a filter paper. (Typically, this mechanical renewal was made before each new experiment or a set of experiments.)

2.3. Analytical procedure

The three electrodes were immersed in to the 10 mL electrochemical cell, containing 0.05 M phosphate buffer (pH 8) medium. Cyclic voltammetric experiments were performed over the potential range of -0.2 to 0.8 V in different scan rates. Differential pulse voltammograms were recorded in amplitude of 150 mV and E_{step} and pulse time were equal to 5 mV and 10 ms respectively. Chronoamperometry experiment was carried out in 0.05 M phosphate buffer (pH 8) medium containing 40 µM of BR in step potential of 0.3 V and interval time of 0.05 s.

3. RESULTS AND DISCUSSION

3.1. Voltammetric behavior of bilirubin on carbon paste electrode

Fig. 1 (A and B) are showing the structures of bilirubin and its oxidation product as well as corresponding cyclic voltammogram at carbon paste electrode. Oxidation process of bilirubin includes two steps; oxidation of bilirubin to biliverdin (peak I) and biliverdin to purpurin (peak II). As can be seen here and it was reported before [39], anodic oxidation of BR at carbon paste electrode is a typically redox transformation, giving rise to the peak at nearly +0.32 V. It also showed that no reduction peak was observed in the reverse scan, suggesting that the electrochemical reaction is a totally irreversible process.

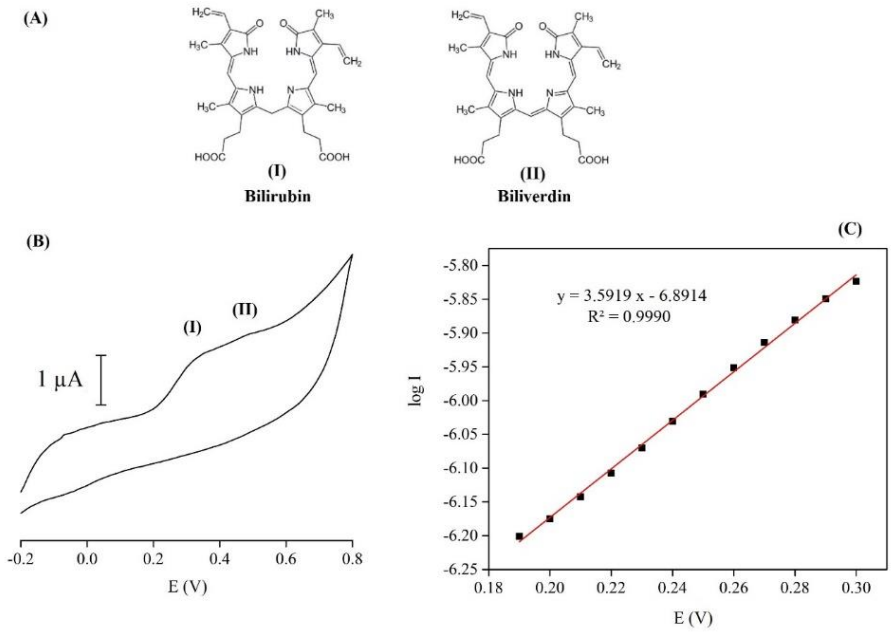

Fig. 1. Chemical structure of bilirubin and biliverdin (A), representative cyclic voltammogram of the bilirubin oxidation process in two steps (B) and corresponding Tafel plot (C); (analysis condition: PBS 0.05 M, pH 8, CV scan rate=30 mV/s)

For totally irreversible systems, nα can be calculated according to the following equation [40].

$$n\alpha = \frac{47.7}{E_P - E_{P/2}} \quad (1)$$

where $E_{p/2}$ is the potential where the current is at half the peak value. So, from this equation the value of nα was obtained as 0.48.

Tafel plot as an accountable way to calculate the electron transfer coefficient (α) was also demonstrated in Fig. 1 C according to equation (2);

$$\log I = \log I_0 + \frac{(1-\alpha)nF}{2.3\,RT} E \quad (2)$$

where R (8.314 J/K mol) is the gas constant, T (298 K) is room temperature, F (96485 C/mol) is Faraday constant, α is the electron transfer coefficient, and n is the number of exchanged electron. With this calculation, α was obtained as 0.71.

3.2. Chronoamperometry experiment (surface area and diffusion coefficient calculation)

In order to measure the electrochemically active surface area (A) of the carbon paste electrode, the chronoamprogram of 0.1 mM potassium ferrocyanide as the redox probe was recorded. In chronoamperometric studies, the current for the electrochemical reaction of ferrocyanide (at a mass-transfer-limited rate) that diffuse to an electrode surface is described by the Cottrell equation:

$$i = \frac{nFAC^* D^{1/2}}{\sqrt{\pi t}} \tag{3}$$

where A is the electrochemical surface area, D is the diffusion coefficient, C^* is the bulk concentration of ferrocyanide and the other parameters have their usual meanings. Under diffusion control, a plot of i versus $t^{-1/2}$ will be linear and from the slope, the value of A can be obtained, since the precise value of the diffusion coefficient of ferrocyanide is well known (6.20×10^{-6} cm^2 s^{-1}). The electrochemically active area of the unmodified carbon paste electrode was 0.04 cm^2.

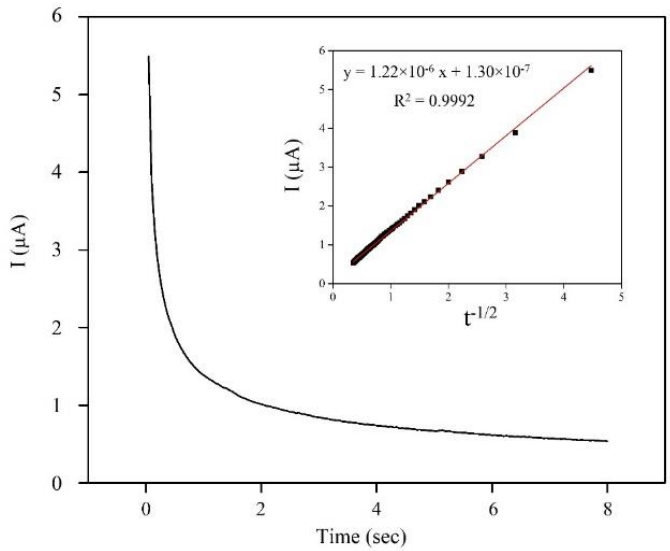

Fig. 2. Chronoamperogram of bilirubin (40 µM) in phosphate buffer (0.05 M, pH 8) at the E=0.3 V; (inset is showing the current signals *vs.* $t^{-1/2}$)

In the next step, the diffusion coefficient of bilirubin on the surface of carbon paste electrode was obtained by the same calculation. In order to this aim, the chronoamprogram of 40 µM bilirubin in phosphate buffer (0.05 M, pH 8) at the step potential of 0.3 V was recorded.

Considering the (A) equal to 0.04 cm^2, (D) is obtained 1.4×10^{-5} $cm^2\ s^{-1}$ for this irreversible one-electron reaction. Fig. 2 and inset indicate the corresponding chronoamperogram and linear relationship of i *vs.* $t^{-1/2}$ respectively.

3.3. Scan rate investigation

Cyclic voltammetry was carried out to analyze the influence of scan rate on oxidation of BR at CPE. The results shown in Fig. 3 are from CPE electrode in 0.05 M PBS (pH 8) containing 40 µM BR at scan rates over the range of 0.01–0.30 V s^{-1}. It is obvious that the oxidation peak currents are improved upon raising the scan rate (Fig. 3 A). The linear relationship of I (µA) versus scan rate (ν) and square root of scan rate ($\nu^{1/2}$) is demonstrated as Fig.3 B and C, respectively.

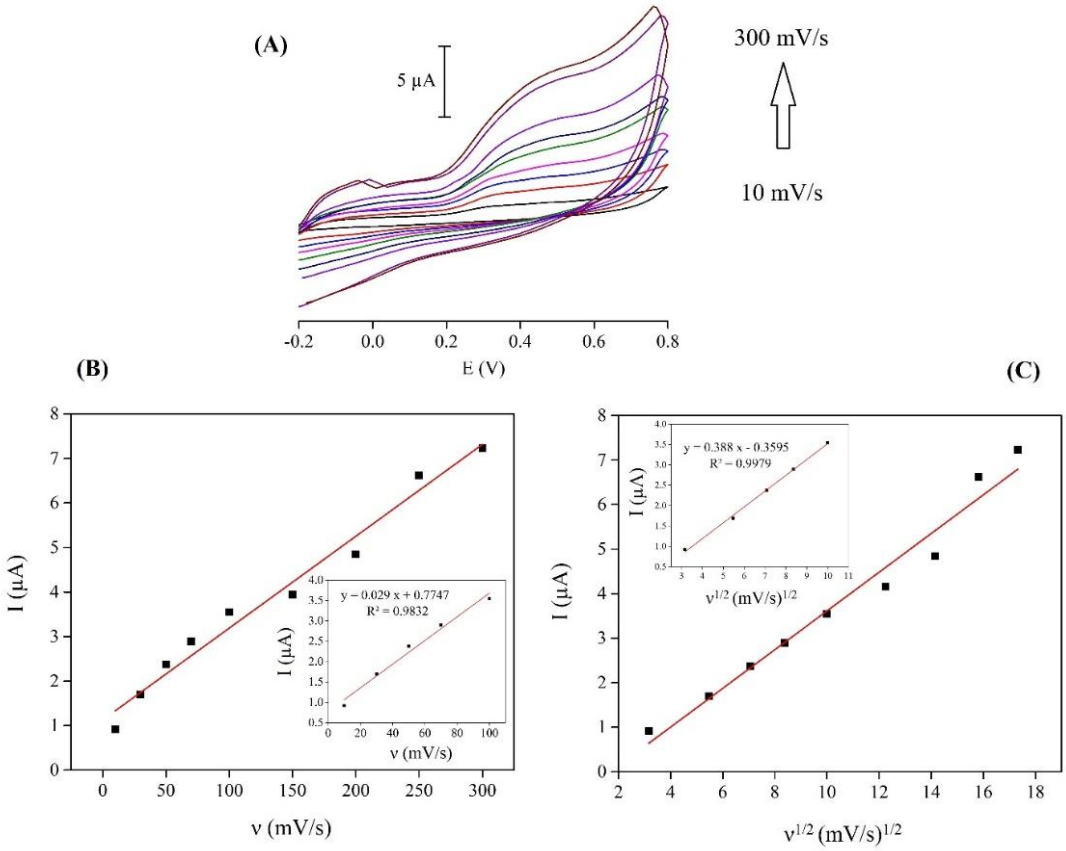

Fig. 3. cyclic voltammetry responses of BR (40 µM) obtained using CPE at various potential scan rates including: 10, 30, 50, 70, 100, 150, 200, 250 and 300 mVs^{-1}(A); the plot of peak current *vs.* υ (B) and peak current versus $\upsilon^{1/2}$ (C)

Comparison of the regression coefficients in this two modes, suggests that the oxidation process of BR follows diffusion–controlled mechanism at the surface of CPE and this behavior is more obvious in the scan rates below 0.1 V s^{-1} which is expressed by the linear regression equation as: I (µA)=0.388 $\nu^{1/2}$ – 0.3595 (R^2=0.9979). In addition, a plot of logarithm of the anodic peak current (A) *vs*. logarithm of scan rate (mV s^{-1}) gave a straight line with a slope of

0.59 with R^2=0.9991 (graph is not shown here) near the ideal theoretic value of 0.5, confirms the diffusion controlled mechanism [41].

3.4. Investigation of pH effect on electrode signal

The pH dependence of anodic peak potentials and anodic peak currents for BR on the carbon paste electrode was examined in the pH range of 6 to 10 (Fig. 4 A). It was found that raising the pH in this range, leads to a negative shift in the oxidation peak potentials of BR. Fig. 4 B indicates the dependence of oxidation peak potential of BR to pH and the protons involvement in the electrode reaction. The pH dependence of E_{pa} can be expressed by the linear regression equations as: E_{pa}=−0.060 pH+0.772 (R^2=0.983) for BR. Based on Nernst equation (0.059 m/n), the slope of −0.060 V pH^{-1} suggests that in the electrode reaction process, the numbers of protons and electrons is in the equal number.

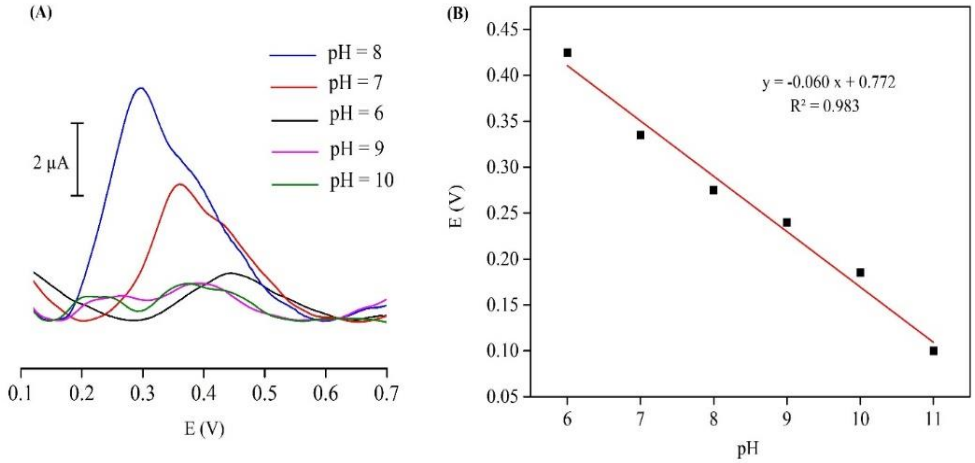

Fig. 4. Differential pulse voltammetry signals of the CPE for 20 µM of BR in different pHs (A) and linear relationship of pH *vs.* peak potential (B); (DPV parameters: pulse time=10 ms, amplitude=100 mV, E_{step}=5 mV)

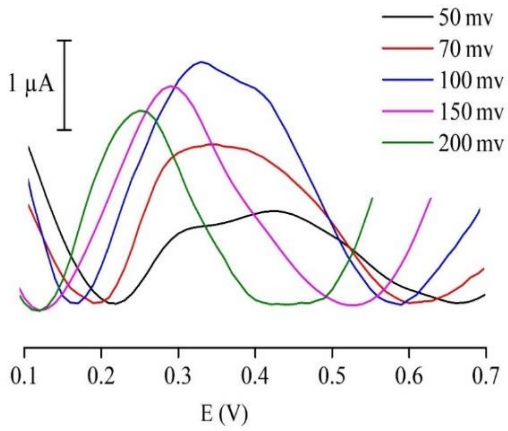

Fig. 5. Comparison of DPV responses of CPE in BR (10 µM) for different pulse amplitudes; (DPV parameters: pulse time=10 ms, E_{step}=5 mV)

3.5. Pulse amplitude optimization

One of the instrumental parameters which can be optimized to achieve higher current signals, is pulse amplitude. In order to this optimization, CPE signal was recorded in 10 µM of BR at different pulse amplitudes. As can be seen in Fig. 5 the amplitude of 150 mV can be the optimum value both in terms of peak shape and peak height together.

3.6. Calibration curve

Fig. 6 shows the calibration curve and voltammograms of different concentrations of BR for the proposed sensor under optimum conditions. As can be seen, there is a linear relationship between the oxidation current signal and the BR concentration in the range of 3.5 to 25 µM. This electrode shows a linear region in the mentioned concentration range with the desired value of the correlation coefficient. The detection limit obtained for this method was 1.2 µM.

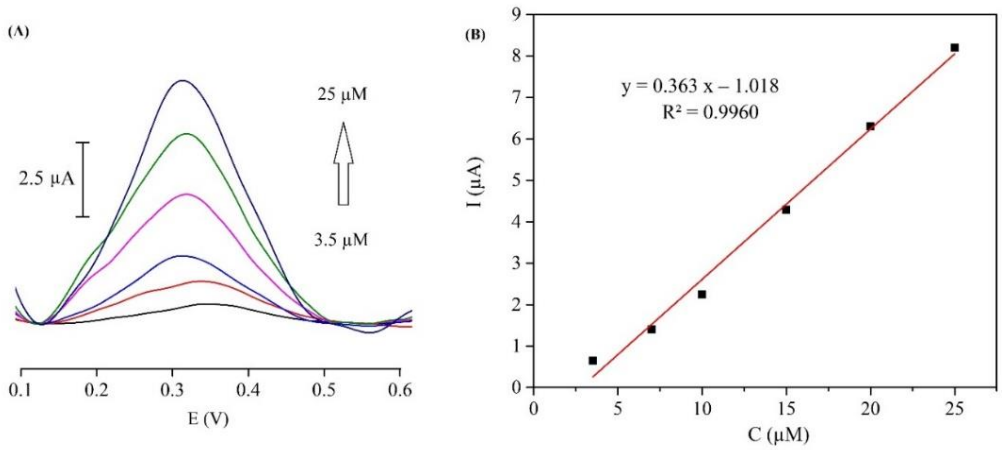

Fig. 6. Differential pulse voltammograms obtained from different concentrations of BR using CPE (A) and calibration curve (B) in PBS pH=8 at optimum conditions; pulse time=10 ms, amplitude=150 mV, E_{step}=5 mV

4. CONCLUSION

This work has investigated the electroactivity of a conventional carbon paste electrode towards bilirubin. Two step oxidation process was recognized by cyclic voltammetry which were occurred at ≈300 mV and ≈500 mV, respectively. These reactions were found to be highly irreversible.

The transfer coefficient (α), number of transferred electrodes (n) and diffusion coefficient (D) were calculated from cyclic voltammetric and chronoamperometric responses. In the following, the optimum pH for measurement of BR was determined and the calibration curve was plotted under optimum conditions. The proposed method does not require expensive instruments or critical analytical reagents.

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