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ELECTROCHEMICAL BEHAVIOUR OF TOLUIDINE BLUE O COVALENTLY MODIFIED MICROCYLINDER CARBON FIBER ELECTRODE AND AMPEROMETRIC DETERMINATION OF HEMOGLOBIN IN WHOLE BLOOD

Keywords microelectrode, chemically modified electrode, electrocatalysis, amperometry, hemoglobin, whole blood.

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ABSTRACT

The hemoglobin concentration in diluted whole blood has been amperometrically measured using a toluidine blue O covalently modified microcylinder carbon fiber electrode. The modified electrode was prepared by modifying the surface of carbon fiber by cyclic voltammetric oxidation to form a lot of -COOH groups, and then covalently linking toluidine blue O (TBO) by acylation. The surface morphology and stability of the TBO modified electrode are discussed. The electrochemical behaviour of the modified electrode was studied by cyclic voltammetry. The number of H^+ participating in the surface electrode process and some kinetic parameters in various pH solutions were determined. The TBO modified microelectrode showed significant

electrocatalytic activity for the oxidation of hemoglobin(HB) in pH 5.4 solution. The amperometric response to HB increased linearly with increasing concentration from $8.0x10^{-6}$ to $8.0x10^{-4}$ M. The obtained HB concentration correlated well with that obtained using a reference procedure.

INTRODUCTION

Recently, much study in analytical fields has focused on the application of microelectrodes and assays in biochemical analysis[1,2]. The modification of microelectrodes has extended the application range[2-7]. However, because of the high mass transport rate of the microelectrodes[1,8], the modifier on the electrode surface prepared by irreversible adsorption rapidly diffuses away from the electrode surface in the process of electrochemical reaction, thus greatly decreasing the catalytic efficiency and stability. Thus, the preparation of modified carbon fiber microelectrodes (CFME) has usually been conducted by using electrochemical polymerization, use of a coating polymer[6,9] or noble metal deposition[7] to fix the mediator efficiently. One possibility is covalent modification by acylation with suitable molecules acting as redox mediators on the surface of a carbon electrode[10,11]. Albery et al[12] outlined the method in a review. However, to our knowlege, up to now the method has only been used to immobilize enzyme at a carbon fiber microelectrode by using carbodiimide[13,14], and the work on covalently modifying a CFME with a dye mediator by direct amidation with thionyl chloride has not been reported. In this work, the carbon fiber surface was electrochemically pretreated to increase the oxide density[13,14] and to form many acid and ester groups. The -COOH groups formed were activated with thionyl chloride to form -COCl groups, and then were very easy amidated with a dye mediator, toluidine blue O (TBO), which can be used as a electron transfer mediator[15,16]. The TBO modified graphite and glassy carbon electrodes formed by irreversible adsorption can catalyze the oxidations of both

NADH[15] and hemoglobin[16] . Here the TBO covalently modified carbon fiber microelectrode can also catalyze the oxidation of hemoglobin.

As is well known, hemoglobin (HB) is an impormant respiratory protein in red cells, being a carrier of O_2 transference. The direct electrochemical determination of HB, particularly in a small system, is very significant in clinic medicine[6]. Some works on the direct electrochemical determination of hemoglobin have been reported[6,16,17]. However, little work on the direct electrochemical determination HB at a microelectrode has been studied, and the amperometric detection of HB with a carbon fiber microelectrode has not been reported. The result could suit the needs and challenges of miniaturization of detectors and detection devices, and would be significant in development of amperometric microbiosensors.

EXPERIMENTAL

Reagents

Toluidine Blue O(B.S.) was obtained from the Chroma Chemical Reagent Company(Germany), hemoglobin (from ox blood) was from Shanghai Biochemistry Research Institute of China. The reagents used to prepare both pH 2-12 phosphate buffer solutions and pH 5.4 acetate buffer solution, thionyl chloride($SOCl_2$) and other reagents were of analytical reagent grade. Pyridine solvent was redistilled with P_2O_5(8 g per 100 ml) and was stored over a 4A molecular sieve activated at 550°C Water used in experiment was twice-quartz -distilled. Carbon fiber (PAN type) with 6-7 um diameter came from the Shanghai Synthetic Fiber Research Institute. Epon 812 epoxy resin (New York, U.S.A) was used to seal the electrodes.

Equipment

Electrochemical measurements were carried out with a BAS-100B Electrochemical Analyzer equipped with a PA-1 Preamplifier which was used to amplify current and filter out noise, and a FPG-310 Color Plotter (Fujitsu Company, Japan) which was

used to record the voltammograms. Scanning electron micrographs were obtained with a X-560 scanning electron microscope (Hitachi, Japan). The experimental temperature was controlled at 20±0.1°C.

Procedure

The fabrication of the single carbon fiber microcylinder electrode with a length of 6-10mm was usually similar to that given in a previous paper[6]. Briefly, single carbon fiber was sealed in glass capillary tube with epoxy resin and was then cut with a scissors to a needed length. After the carbon fiber microcylinder electrode was washed thoroughly with acetone and distilled water, it was electrochemically pretreated in fresh 1.0 M H_2SO_4 solution with a triangular-wave potential sweep from -1.0 V to +2.0 V at the scan rate of 200 mV s^{-1} for 50 minutes. The pretreated electrode was immersed in $SOCl_2$ for 30 minutes, and then rinsed with tetrahydrofuran(THF) to take out the remaining $SOCl_2$ from the electrode surface. Finally, it was dipped in anhydrous pyridine solution containing $1.0x10^{-3}$ M toluidine blue O for 15 minutes, and then was rinsed with phosphate buffer(pH 7.0) and stored in the same buffer solution.

A three-electrode system with a saturated calomel electrode (SCE) as reference, Pt wire as counter and above modified electrode as working electrode was employed. After deaerating with pure N_2 for 10 minutes, the electrochemical measurements were performed under a nitrogen atmosphere inside a Faraday cage.

RESULTS AND DISCUSSION

Surface Morphology and Stability of TBO Modified Microelectrode

The process of electrode modification was viewed with the electron scanning microscope. Fig.1 shows the microscope graphs, which were obtained by cutting the carbon fibers at various stages of the modifying process on the sample stand, then treating them by spraying gold, then amplifying by 12,000 times. On the surface of a

unpretreated carbon fiber, there was a lot of explicit stripes, but in the Y-axis direction it was smooth (Fig.1A). However, after electrochemical pretreatment, the stripes disappeared (Fig.1B), many embossed sites appeared, and the surface became rather rough but more uniform than that of unpretreated carbon fiber. Comparing Fig.1B with 1C, some difference occured on the surface acylated with $SOCl_2$ by dipping the electrode in $SOCl_2$ for 30 minutes. After the acylated fiber was dipped in an anhydrous pyridine solution of TBO for 15 minutes, the surface showed a uniform cloudiness, indicating that the acylated carbon fiber surface was aminated by the reaction between -COCl and TBO. There also was some TBO adsorbed on the fiber surface.

The repetitive sweeps of cyclic voltammetry indicated that at the beginning of sweep both the cathodic and the anodic peak currents rapidly decreased, dropping to a constant value which remained constant for a very long time. After the modified microelectrode was dipped in pH 7.0 buffer solution for several weeks, no change of peak currents appeared, indicating that the stability of TBO modified carbon fiber microelectrode was very good. The decrease of peak currents at the beginning was due to the desorption of the TBO adsorbed on the electrode surface. The reaction in the process of electrode modification can be shown in Fig.2. TBO was covalently bound to the electrode surface, greatly strengthening the stability of modified electrode.

Kinetic Behaviour of the TBO Modified Electrode

Fig.3 shows that not only the cyclic voltammetric peak currents of the TBO modified electrode are proportional to the scan rate v, but also the peak potentials shift with increasing of scan rate. Thus the electrode process is controlled by surface concentration and electron transfer rate of TBO on the electrode. With increasing lnv, the anodic peak potential shifted linearly in the positive direction, and the cathodic peak potential shifted linearly in the negative direction with a slope of 23.3 mV eg.Fig.3 for $E_{p,c}$. From the slope, the number of electron transfer of 2 [15] and the equation of $E_{p,c}$= constant -(0.5RT/αnF)lnv, the charge transfer coefficient α=0.53 can be obtained.

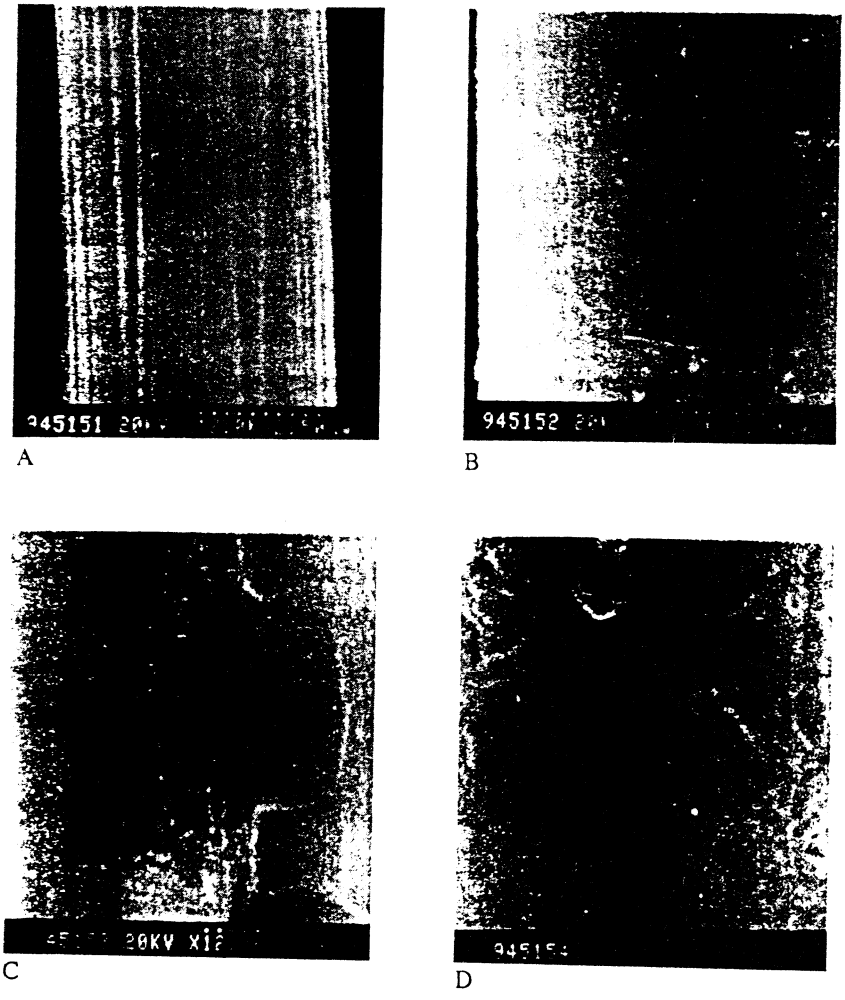

FIG.1 The scanning electron micrographs of A. unpretreated, B. electrochemically pretreated, C. acylated, and D. aminated carbon fiber.

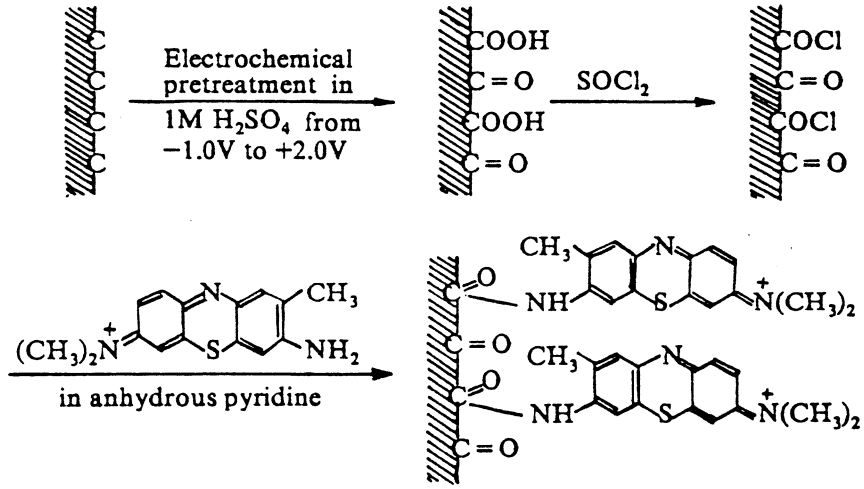

FIG.2 The Schematical diagram for the preparation of the TBO modified electrode.

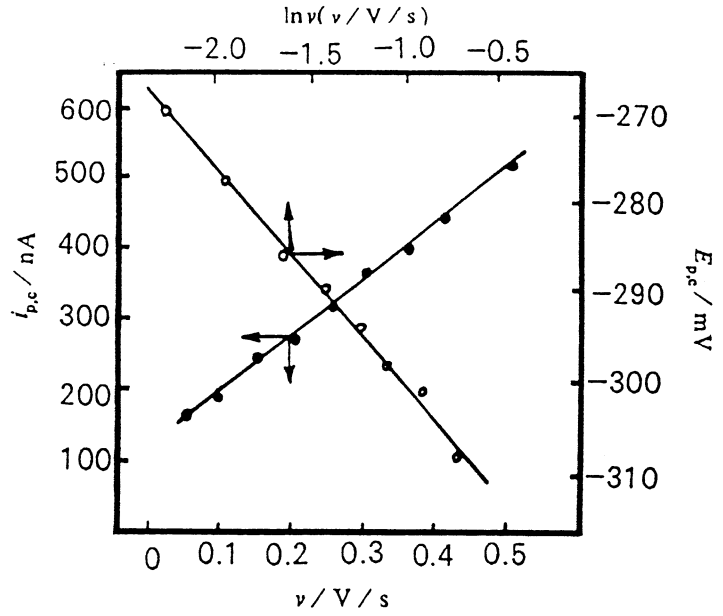

FIG.3 The plots of $i_{p,c}$ vs. v for (•) and $E_{p,c}$ vs. lnv for (○) at the TBO modified electrode in pH 7.0 buffer solution.

At the TBO modified electrode, with the increasing of pH in the range of 2 to 11, the peak potentials of both the anodic and the cathodic peaks shifted in the negative direction. The normal formal potential, $E^{o'}$, which was obtained from α of 0.53 and $E^{o'}=E_{p,a}-a(E_{p,a}-E_{p,c})$[19], shifted in a negative direction with change in slope at pH 6.5 with increase of pH, as shown in Fig.4. $E^{o'}$/pH was -63 mV/pH at pH<6.5 and -32 mV/pH at pH>6.5, respectively. Thus, the number of H^+ participating in this electrode process is 2 at pH<6.5 and 1 at pH>6.5.

However, in the buffer solutions (pH 1.5-11) including $1.0x10^{-4}$M TBO, the cyclic voltammograms of a fresh bare carbon fiber electrode indicated that the peak potentials of TBO shifted in a negative direction with two changes in slope at pH 3.7 and 5.5 [20] (Fig.4), the three slopes were -87, -58 and -29 mV/pH° at 20 C, respectively; the number of H^+'s participating in the electrode process of TB in solution is 3 at pH<3.7, 2 at 3.7<pH<5.5 and 1 at pH>5.5. In reference [21] pK_{r1} and pK_{r2}, which are the first and second ionization constants of hydrogen ion at each of the amino groups of TBO in the reductant, are 4.81 and 5.41 for TBO at 20°C, respectively. The pk_{r2} is very close to the value of 5.5.

At higher pH's, the results of TBO bound to the electrode and TBO in solution are similar. But at lower pH's, the numbers of H^+'s participating in the electrode process are different because when TBO is acylated the "N" atom covalently bound to -C=O group is hardly possible to be protonized due to the conjugation between p orbital in "N" atom and sp^2 carbon orbital in -C=O group, which reduces the electronegativity and protonaffinity of the "N" atom. Only other two "N" atoms in acylated TBO can be protonized at lower pH. However, all three "N" atoms in free TBO can be protonized[21]. These results also indicated that TBO at the modified electrode is acylated.

From the equation of Laviron[18]: $\log k=\alpha\log(1-\alpha)+(1-a)\log\alpha-\log(RT/nFv)-\alpha(1-\alpha)nF\Delta E_p/2.3RT$, the value of α (0.53) as well as ΔE_p of cyclic voltammograms at various pHs, the apparent surface electron transfer rate constant k can be obtained. The results indicated that the effects of pH on ΔE_p and k were very complicated. The whole electrode process was affected by two factors: the electrode reaction of TBO

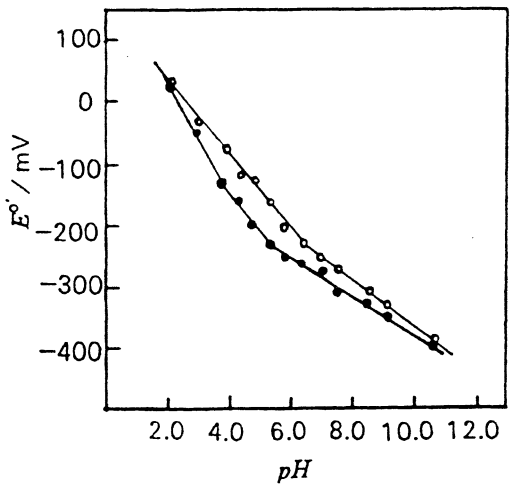

FIG.4 Dependence of E#$^{o'}$ of TBO covalently bound to carbon fiber electrode (o) and TB in solution (•) on pH.

with the participation of H^+ and the electrochemical activation of carbon fiber surface with high density of surface oxygenous groups. When the pH was very low, the electrochemical activation of the electrode surface was smaller, k being very small(eg. $0.94s^{-1}$ at pH 2.1). When 3.1<pH<7.0, the k value varied slightly between 1.30 and 1.65. However, when the pH>7.0, the surface activation did not change with increasing of pH, but it was disadvantageous to the electrode reaction with the participation of H^+, thus, k decreased.

Electrocatalytic oxidation of hemoglobin at the TBO modified electrode

Fig.5 shows the electrocatalysis of the TBO modified electrode to the oxidation of HB in pH 5.4 acetate buffer solution. When the buffer solution included $4.0x10^{-4}$M HB, the anodic peak current increased, the cathodic peak current decreased, and the anodic peak potential shifted in a positive direction for about 30mV. Thus the

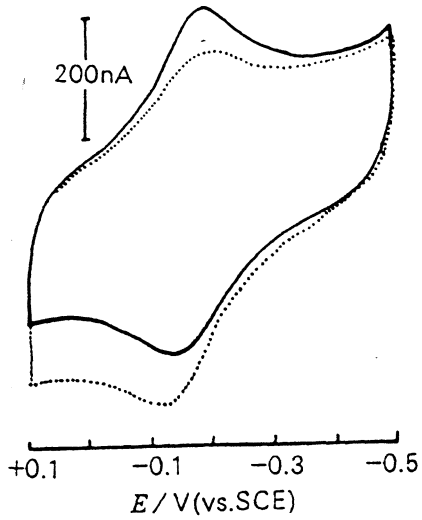

FIG.5 Cyclic voltammograms of the TBO modified electrode in pH 5.4 buffer solution (_) and pH 5.4 buffer including 1.0×10^{-4}M HB(...) at v=100mV/s.

catalytic reaction between bound TBO and HB in solution possibly changes the electron transfer rate between electrode and TBO at the same potential. The catalytic mechanism can be expressed as follows:

$$TBO_{red} - 2e \rightleftharpoons TBO_{ox}$$

$$2HbFe(II) + TBO_{ox} \rightarrow TBO_{red} + 2HbFe(III)$$

The result was consistent with previous work at a polymer modified electrode[6]. However, it was different from the one at a methylene blue modified carbon electrode by irreversible adsorption[17], in which the modified electrode was able to catalyze the reduction of HB, and the peak potential shifted in a negative direction. It is suggested that the catalytic mechanism at a covalently bound or polymer modified electrode can be different from that at a adsorbed modified electrode.

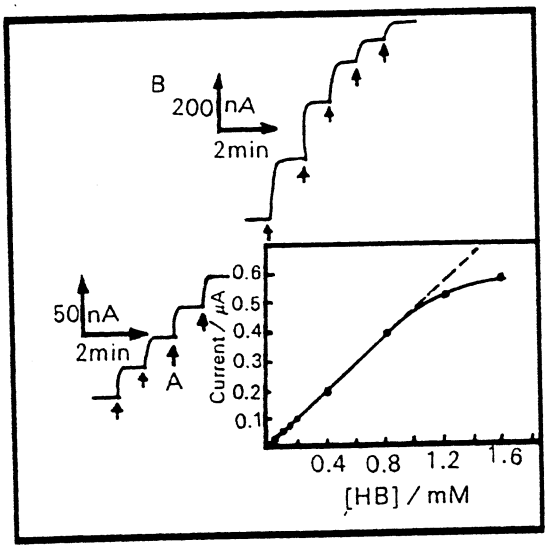

FIG.6 Amperometric response of the TBO modified microelectrode to successive increments of 50μM (A) and 0.4mM (B) HB with an interval of 1 min at -0.12V and pH 5.4.

Amperometric Response of the TBO Modified Electrode to Hemoglobin

The amperometric experiments at various operating potentials indicated that the modified electrode had an amperometric response to HB at the more positive potential than -0.3V in weak acid solution. With the positive shift of applied potential, the response rose sharply up to -0.1V, and then exhibited a constant value. The amperometric response to HB was seriously affected by the pH value of the solution. With increasing pH, the response increasing in the range of pH 2.1 to 5.0, remained a constant value at pH 5.0 to 6.2, then decreased. Therefore, the amperometric determination was performed at -0.1V and in pH 5.4 buffer solution.

In pH 5.4 solution, with the injecting of HB, the amperometric response current increased. The curve of steady state response is shown in Fig.6. It can be seen from Fig.6 that the response time is very short at lower concentrations of HB and the response has a linear increment with increasing concentration of HB in the range of

$8.0x10^{-6}$ to $8.0x10^{-4}$M with a correlation coefficient of 0.987(see Fig.6). The average current response to a HB concentration increase of $1.0x10^{-5}$M was 5.2nA(SD=6.3%) for five successive determinations. When the HB concentration was larger than $8x10^{-4}$M, the response time became long, the current also deviated from linearity. Moreover, the lifetime of the modified electrode shortened. In a solution of HB concentration lower than $4.0x10^{-4}$M, the electrode can be successively used for five days. This is very advantageous for clinical analysis of HB.

Amperometric Determination of Hemoglobin in Clinic Blood

After a 20μl clinic blood sample was 100-fold diluted with pH 5.4 buffer solution, the amperometric response of the TBO modified electrode can be used to detect the concentration of HB at -0.1V. The average value of three determinations in an interval two hours was $1.98x10^{-5}$M(SD=3.6%). Thus, the concentration of HB in whole blood is 1.98mM(=129g/L). The result was close to the value of 131g/L obtained with a red blood corpuscle meter. The TBO covalently modified carbon fiber electrode could be used quite well in a clinical determination.

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