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Electrochemical Biosensor Based on Hemoglobin and Titanate Nanotubes Modified Electrode and its Application

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An electrochemical biosensor based on hemoglobin (Hb) and titanate nanotubes (TNTs) modified carbon ionic liquid electrode (CILE) was prepared with direct electrochemistry and electrocatalysis of Hb investigated. The morphology and structure of TNTs was analyzed by field emission scanning electron microscopy and transmission electron microscopy. UV-Vis and FT-IR spectra confirmed that Hb maintained the natural conformation with TNTs. A pair of enhanced reversible redox peak could be observed on Nafion/Hb/TNTs/CILE, indicating that the positive effects of TNTs to the electron transfer. The electrochemical parameters of Hb on the modified electrode were calculated with the number of electron transfer (n), the charge transfer coefficient (α) and the electrode reaction rate constant (k_s) obtained as 1.18, 0.478 and 0.85 s^{-1}, respectively. The electrochemical sensor was applied to investigate the electrocatalytic performance to trichloroacetic acid (TCA) and nitrite with excellent ability.

Keywords: hemoglobin; titanate nanotubes; direct electrochemistry; electrocatalysis.

1. INTRODUCTION

Hemoglobin (Hb) is a typical redox protein with large three-dimensional structure consisting of two α-polypeptide and two β-polypeptide chains [1], which is an essential biological molecule with important role in the life process of organisms such as energy conversion and metabolism process. Also Hb has an ideal electroactive center and can be used for electrochemical biosensor to investigate the electrocatalytic reduction of various substrates such as bromate, sodium nitrite, hydrogen peroxide

(H_2O_2) and trichloroacetic acid (TCA) [2-5]. The properties of electrochemical biosensor based on redox protein can be investigated by different electrochemical techniques including electrochemical impedance spectroscopy (EIS), differential pulse voltammetry (DPV) and cyclic voltammetry (CV). However, electrochemical center of Hb is deeply buried in non-conductive peptide chain and direct electron transfer of Hb to the surface of the electrode is difficult to realize. To enhance electrochemical response of Hb on the electrode, many mediators and promoters have been used. Zhang prepared a AuNPs modified ITO electrode to immobilize Hb for the reduction of H_2O_2 [6]. Vilian designed a functionalized multi-walled carbon nanotube, poly-l-histine, and ZnO nanocomposite film for the immobilization of Hb with H_2O_2 and bromate detected in real samples [7]. Xu synthesized a biocomposite film for sensing H_2O_2 based on Hb, graphene, and zinc oxide [8]. Han used monolayer-protected gold nanoparticles for direct electrochemistry of Hb [9].

Titanate nanotubes (TNTs) have rolled two-dimensional nanostructures with enormous unique physicochemical properties such as great mechanical properties, electronic conductivity, open tubular inner pores and high specific surface area [10]. Furthermore, TNTs have been acted as carrier and substrate to immobilize various catalysts and bio-molecules such as enzymes [11], nanoparticles [12] and biomaterials [13]. The synthesis approaches of TNTs include alkaline hydrothermal method [14], electrochemical anodic oxidation [15] and the chemical template assisted method [16].

In this study, TNTs were used to provide a biological microenvironment for Hb to realize the direct electrochemistry on the modified electrode. Nafion is used to form an ion-exchange polymer film on the electrode surface to remain the bioactivity of Hb and ensure the stabilization of electrode. Electrochemical performances of Nafion/Hb/TNTs/CILE were investigated by CV, which showed an enhanced reversible peaks and indicated that TNTs acted as a promoter to accelerate electron transfer. Furthermore, the fabricated biosensor was applied to study the electrocatalysis reduction of TCA and nitrite with satisfactory results.

2. EXPERIMENTAL

2.1 Reagents

Hemoglobin (Hb, Sigma-Aldrich Co., USA), titanate nanotubes (TNTs, Nanjing XFNANO Materials Tech. Ltd. Co., China), 1-hexylpyridinium hexafluorophosphate ($HPPF_6$, Lanzhou Yulu Fine Chem. Ltd. Co., China), graphite powder (particle size 30 μm, Shanghai Colloid Chem. Co., China), Nafion (5% ethanol solution, Sigma-Aldrich Co., USA), trichloroacetic acid (TCA, TianJin Kemiou Chemical Ltd. Co., China), sodium nitrite ($NaNO_2$, Yantai Sahe Chem. Ltd. Co., China) and medical facial peel (35%, Shanghai EKEAR Bio. Tech. Ltd. Co., China) were used as received. The supporting electrolyte was 0.1 mol L^{-1} phosphate buffer solutions (PBS). All the other reagents were of analytical grade and doubly distilled water was used to prepare the solutions.

2.2 Apparatus

Electrochemical measurements were performed by CHI 604B electrochemical workstation (Shanghai CH Instrument, China). A three-electrode system was used for the electrochemical experiments with Nafion/Hb/TNTs/CILE as the working electrode, platinum wire electrode as the auxiliary electrode and saturated calomel electrode (SCE) as the reference electrode. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were performed by JSM-7100F scanning electron microscope (JEOL, Japan) and JEM 2010F instrument (JEOL, Japan). Fourier transform infrared spectroscopy (FTIR) and UV-Vis spectroscopy were carried out using Nicolet 6700 FT-IR spectrophotometer (Thermo Fisher Scientific Inc., USA) and TU-1901 double beam UV-visible spectrophotometer (Beijing General Instrument Ltd. Co., China).

2.3 Construction of electrochemical biosensor

According to references [17,18], CILE was prepared by utilizing $HPPF_6$ and graphite powder, which was polished to mirror-like surface before use. 8 μL the suspension solution of TNTs (1.0 mg mL^{-1}) was decorated on the surface of CILE to get TNTs/CILE. Next 8 μL of Hb solution (15.0 mg mL^{-1}) was dropped onto the surface of TNTs/CILE. After drying, Hb/TNTs/CILE was further modified with 6 μL of Nafion solution (0.5%), which could form a thin film and the modified electrode was denoted as Nafion/Hb/TNTs/CILE. For comparison, Nafion/Hb/CILE, Nafion/TNTs/CILE and Nafion/CILE were also fabricated using a similar procedure.

3. RESULTS AND DISCUSSION

3.1 Morphology and structure of TNTs

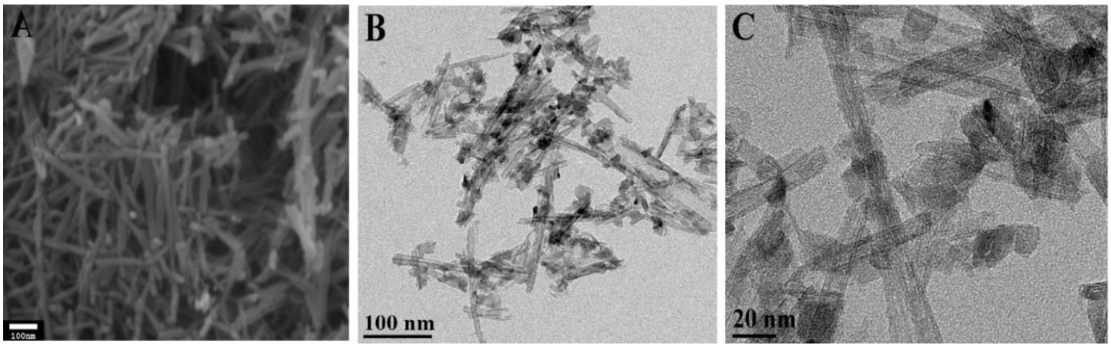

Figure 1. SEM image of TNTs morphology (A), TEM images of TNTs structure at different magnifications (B) and (C).

The structural and morphological characterization of TNTs was recorded by SEM and TEM. Fig.1A shows the SEM image of TNTs with a tubular morphology and multilayer bar-like nanotubes were aggregated together to form a porous nanotubes layer. TEM images (Fig.1B and C) show that

TNTs entangled with each other and an average diameter of 10 nm was got. A single nanotube presents the hollow nature with opening on both sides and a multi-layer wall structure. Therefore, TNTs can provide a large surface and three-dimensional structures on CILE.

3.2 UV-Vis and FT-IR spectroscopic analysis

UV-Vis spectra of Hb and Hb-TNTs mixture are shown in Fig.2A. Soret absorption band of Hb-TNTs mixture was located at 405 nm (curve b), which was the same as that of natural Hb in pH 4.0 PBS (curve a). Therefore, Hb could maintain its natural conformation without denaturation after mixed with TNTs [19,20]. FT-IR is generally used to detect the integrity of the conformation of heme proteins [21,22]. FT-IR spectrum (Fig.2B and C) showed the absorption bands of amide I and amide II of Hb in the presence of TNTs were located at 1652 cm^{-1} and 1540 cm^{-1}, which were basically same as the absorption bands of native Hb (1657 cm^{-1} and 1539 cm^{-1}). The results showed that Hb-TNTs composite membrane retained the original characteristics of secondary structure and did not degenerate during the construction process.

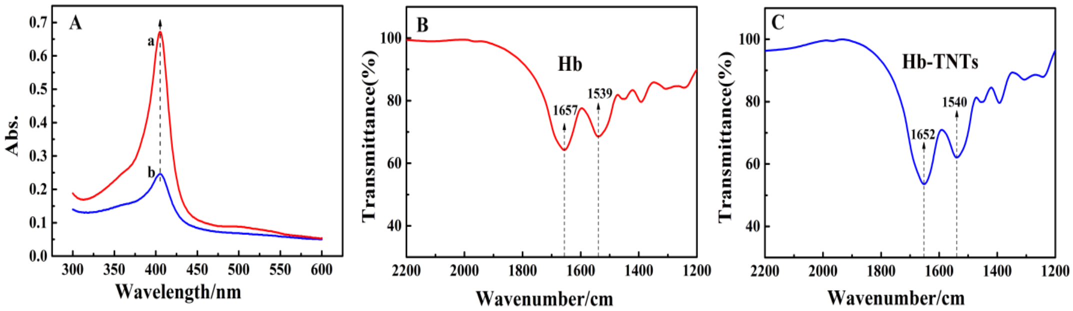

Figure 2. (A) UV-Vis absorption spectra of Hb (a) and Hb-TNTs mixture (b) in pH 4.0 PBS; FTIR spectra of Hb (B) and Hb-TNTs (C) composite film.

3.3 Direct electrochemistry of Hb

Fig.3 shows the cyclic voltammograms of different modified electrodes for direct electrochemical investigation in N_2 saturated pH 4.0 PBS. No electrochemical response could be observed at Nafion/CILE (curve d) and Nafion/TNTs/CILE (curve c). However, on Nafion/Hb/CILE (curve b) a couple of redox peaks displayed, which indicated that direct electron transfer of Hb had been achieved. On Nafion/Hb/TNTs/CILE (curve a) the peak current increased significantly and peak shape was more symmetrical, indicating that the presence of TNTs had a positive effect on accelerating the electron transfer at the electrode surface. The cathodic peak potential (E_{pc}) and anodic peak potential (E_{pa}) was obtained at -0.218 V and -0.139 V (vs. SCE) with formal peak potential ($E^{0'}$) of -0.179 V (vs. SCE). The ratio of redox peak current was close to 1, which was attributed to the redox couple of the active center of Hb (Fe^{3+}/Fe^{2+}) [23,24].

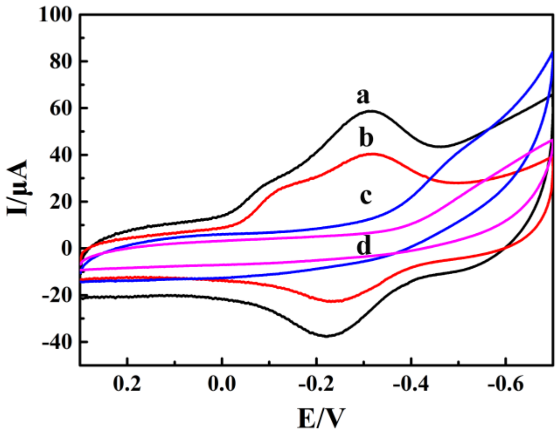

Figure 3. Cyclic voltammograms of (a) Nafion/Hb/TNTs/CILE, (b)Nafion/Hb/CILE, (c)Nafion/TNTs/CILE, (d) Nafion/CILE in pH 4.0 PBS at the scan rate of 0.1 V s^{-1}.

3.4 Electrochemical investigations

Cyclic voltammetric responses of Nafion/Hb/TNTs/CILE at different scan rates were further tested to investigate the direct electrochemical behavior of Hb with the results shown in Fig.4A. With the increase of scan rates, a good symmetrical redox peaks appeared in the range of 100-1000 mV s^{-1} and the redox peak current (Ip) had a good linear relationship with the regression equations (Fig.4B) as Ipa (μA) = -189.96 υ (V s^{-1}) - 20.75 (n = 10, γ = 0.993) and Ipc (μA) = 200.25 υ (V s^{-1}) + 13.80 (n = 10, γ = 0.991), respectively. The result demonstrated a surface-controlled electrochemical process. The relationships of redox peak potential (Ep) with lnυ were constructed with two linear regression equations (Fig.4C) got as Epa(V) = 0.041 lnυ (V s^{-1}) - 0.164 (n=8,γ=0.997) and Epc(V)= -0.046 lnυ (V s^{-1}) - 0.36 (n=8 ,γ=0.994). Based on the Laviron's equations [25], the values of the electron transfer number (n), the charge transfer coefficient (α) and the apparent heterogeneous electron transfer rate constant (k_s) were 1.18, 0.478 and 0.85 s^{-1}, respectively. The value of k_s was higher than some reported values for Hb modified on different substrate electrodes and the compared results were shown in the table 1. The results indicated that TNTs could improve the direct electron transfer rate of Hb due to its long tube and porous structure.

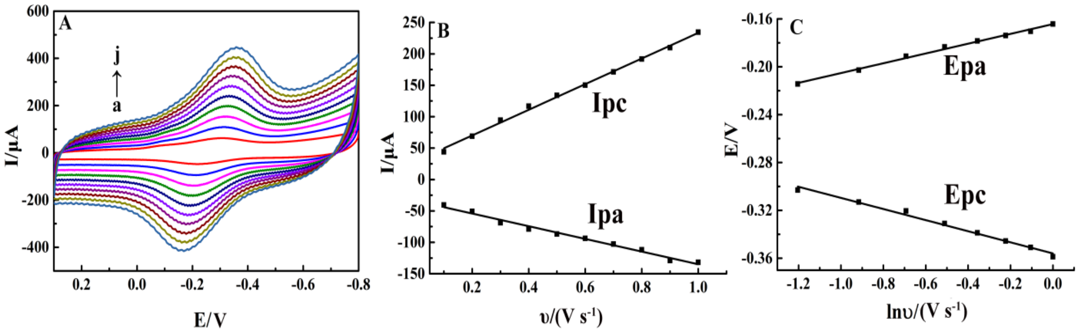

Figure 4. (A) Cyclic voltammograms of Nafion/Hb/TNTs/CILE at different scan rates (from a to j: 100, 200, 300, 400, 500, 600, 700, 800,900,1000 mV s^{-1}) in pH 4.0 PBS, (B) Linear relationship of the redox peak currents versus scan rate(υ), (C) Linear relationship of the redox peak potential versus lnυ.

Table 1. Comparison of k_s with various Hb modified electrodes

Different electrodes	pH	k_s (s^{-1})	References
Nafion/nano-$CaCO_3$/Hb/CILE	4.0	0.75	[27]
Hb/Ag-Ag_2ONPs/silver electrode	7.0	0.239	[28]
Hb/MWNT/GCE	5.4	0.58	[29]
Hb/C_{60}-MWCNT/electrode	7.0	0.39	[30]
Nafion/GR-TiO_2-Hb/CILE	3.0	0.65	[31]
Nafion/Hb/Au/CILE	7.0	0.412	[32]
Nafion/Hb/TNTs/CILE	4.0	0.85	This work

Direct electrochemical behavior of Nafion/Hb/TNTs/CILE was investigated in different PBS with pH range from 3.0 to 8.0, which indicated that the redox peak potential of Hb was negatively shifted with the increase of pH. Therefore, proton was involved in the electrode reaction [26]. The maximum peak current appeared in the pH 4.0 PBS with more stable peak, which was selected as the supporting electrolyte.

3.5 Electrocatalytic behavior

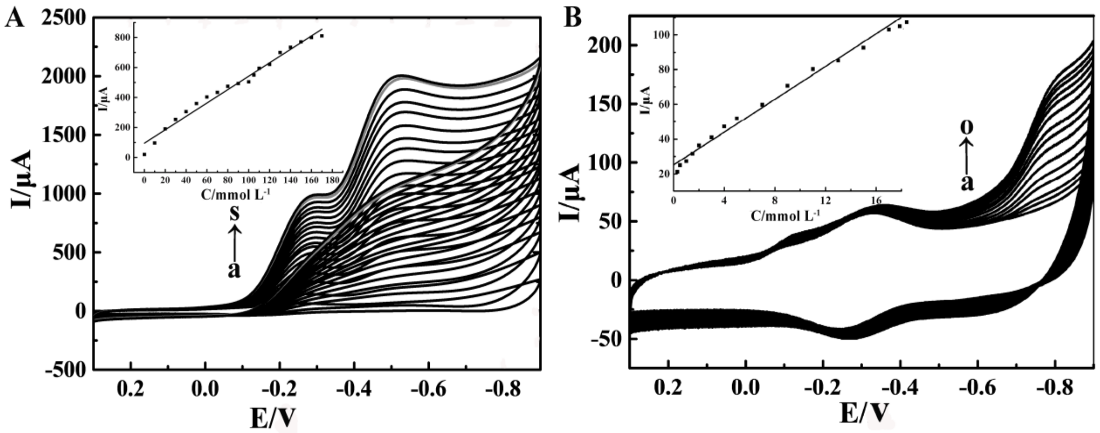

Figure 5. (A) CVs of Nafion/Hb/TNTs/CILE with different concentrations of TCA (from a to s: 0.20, 10.0, 20.0, 30.0, 40.0, 50.0, 60.0, 70.0, 80.0, 90.0, 100.0, 110.0, 120.0, 130.0, 140.0, 150.0, 160.0, 170.0 mmol L^{-1}; inset is the linear relationship of TCA concentration versus reduction peak current); (B) CVs of Nafion/Hb/TNTs/CILE with different concentrations of nitrite (from a to o: 0.3, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 11.0, 13.0, 15.0, 17.0 mmol L^{-1}; inset is the linear relationship of nitrite concentration versus reduction peak current).

Nafion/Hb/TNTs/CILE was applied for the electrocatalytic reduction of TCA and nitrite with cyclic voltammetric results shown in Fig.5. When the increasing concentration of TCA was added in pH 4.0 PBS, an obvious increase in the reduction peak was observed at -0.26 V with decrease of the oxidation peak of Hb (Fig.5A), illustrating that good electrocatalysis of Nafion/Hb/TNTs/CILE to TCA. The catalytic reduction peak current increased linearly with the TCA concentration in a linear range from 0.2 mmol L^{-1} to 170.0 mmol L^{-1} and the linear regression equation was got as I (μA)= 4.44 C（mmol L^{-1}）+ 94.62 (n=19, γ=0.990) with detection limit of 0.067 mmol L^{-1} (3σ). The apparent

Michaelis-Menten constant (K_M^{app}) is an important indicator of reaction kinetics about enzymatic reaction. According to the Lineweaver-Burk equation [33], K_M^{app} was calculated as 122.10 mmol L^{-1}.

As for nitrite, a reduction peak was obtained at -0.780 V with nitrite concentration range from 0.3 to 17.0 mmol L^{-1} (Fig.5B), which could be ascribed to reduction of nitrite with Hb modified electrode [34]. The linear regression equation was I (μA) =4.72C (mmol L^{-1}) + 25.11(n=14, γ=0.994) with the detection limit as 0.083 mmol L^{-1} (3σ). The apparent Michaelis-Menten constant (K_M^{app}) was counted as 2.74 mmol L^{-1}.

3.6 Analytical application

In order to investigate the practical application of Nafion/Hb/TNTs/CILE, the medical facial peel solution and lab water were used as real samples for TCA and nitrite detection with the results shown in table 2. The corresponding recoveries were calculated by the standard addition method with the results in the range from 91.40 % to 103.40 % for TCA and from 99.3 % to 101.6 % for nitrite, indicating that fabricated sensor could be used for the detection of TCA and nitrite in real samples.

Table 2. Detection results of TCA and nitrite samples by the proposed method (n=3).

Sample	Detected (mmol L^{-1})	Added (mmol L^{-1})	Total (mmol L^{-1})	Recovery (%)
Medical facial peel solution	33.19	10.00	42.37	91.80
		20.00	51.47	91.40
		30.00	64.23	103.40
Lab water	0	10.00	10.16	101.60
		20.00	20.74	103.60
		30.00	29.78	99.30

4. CONCLUSION

In this paper, a redox enzyme electrochemical biosensor was manufactured based on Hb immobilized with TNTs. The excellent conductivity with large surface area of TNTs provided an enhanced platform for direct electron transfer between Hb and substrate electrode. Nafion/Hb/TNTs/CILE showed excellent electrocatalytic reduction activity to TCA and nitrite with wider linear range and lower detection limit, proving the potential application of TNTs in the field of electrochemical sensing.

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