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Author: J. Li H. Mei W. Zheng P. Pan X.J. Sun F. Li F. Guo
H.M. Zhou J.Y. Ma X.X. Xu Y.F. Zheng



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A novel hydrogen peroxide biosensor based on hemoglobin-collagen-CNTs composite nanofibers

J. Li^{1*}, H. Mei¹, W. Zheng^{2*}, P. Pan¹, X.J. Sun¹, F. Li¹, F. Guo², H.M. Zhou², J.Y. Ma², X.X. Xu² and Y.F. Zheng^{2,3}

1 Key Laboratory of Green Chemical Engineering and Technology of College of Heilongjiang Province, College of Chemical and Environmental Engineering, Harbin University of Science and Technology, Harbin 150040, China

2 Center for Biomedical Materials and Engineering, Harbin Engineering University, Harbin 150001, China

3 Department of Materials Science and Engineering, College of Engineering, Peking University, Beijing 100871, China

***Corresponding author, Please send all correspondence to**

Prof. W. Zheng,
Center for Biomedical Materials and Engineering, Harbin Engineering University, No.145, Na-Tong-Da Street, Nan-Gang District, Harbin 150001, China
Tel&Fax: 0086-451-8251 8644
E-mail: zhengwei75@126.com

***Co-corresponding author**

J. Li, PhD
Key Laboratory of Green Chemical Engineering and Technology of College of Heilongjiang Province, College of Chemical and Environmental Engineering, Harbin University of Science and Technology, No.52, Xuefu Road, Nan-Gang District, Harbin 150080, China
Tel&Fax: 0086-451-8639 2713
E-mail: lijia@hrbust.edu.cn

Abstract

In this paper, carbon nanotubes (CNTs) were successfully incorporated in the composite composed of hemoglobin (Hb) and collagen using co-electrospinning technology. The formed Hb-collagen-CNTs composite nanofibers possessed distinct advantage of three-dimensional porous structure, biocompatibility and excellent stability. The Hb immobilized in the electrospun nanofibers retained its natural structure and the heterogeneous electron transfer rate constant (k_s) of the direct electron transfer between Hb and electrodes was 5.3 s^{-1} . In addition, the electrospun Hb-collagen-CNTs nanofibers modified electrodes showed good electrocatalytic properties toward H_2O_2 with a detection limit of $0.91 \text{ }\mu\text{M}$ (signal-to-noise ratio of 3) and the apparent Michaelis-Menten constant

(K_m^{app}) of 32.6 μ M.

Keywords: Electrospinning; Hemoglobin-collagen-CNTs nanofibers; Electrochemistry; Biosensor

1. Introduction

In recent years, the electrochemical biosensors based on redox proteins or enzymes have been widely used in various areas such as environmental monitoring, food industry, medical diagnosis, because of their high sensitivity, nice selectivity, repeatability, simple operation and low expense of fabrication [1]. The studies of the third-generation biosensors based on redox proteins or enzymes, which are featured by direct electron transfer of proteins, provide useful information about the thermodynamics and kinetics of the electron transfer process. However, it is usually difficult to accomplish the direct electron transfer on the bare electrode because of the deep burying of the activity center in the polypeptide chain, the high molecular weight and complicated molecular structure of most proteins and enzymes [2]. Many attempts have been made to solve this problem by immobilizing the proteins or enzymes in appropriate matrixes [3-12]. Among these methods, nanomaterials have attracted considerable attention due to their unique properties such as electrical, catalytic, surface effect. Therefore, nanomaterials such as nano-Au [9], nano-Ag [10], TiO₂-graphene nanocomposite [11] and carbon nanotubes-hydroxyapatite [12] have brought new opportunity and wider space for the further development of biosensors. The sensitivity, detection limit and the linear response ranges of biosensors based on nanomaterials have been improved owing to the special properties of nanomaterials.

The electrospinning is a simple and efficient technology for producing one-dimensional nanomaterials, and beneficial to control the size of nanomaterials ranged from nanometer to micrometer [13-16]. Therefore, electrospun nanomaterials have attracted increasing research attention in recent years. The electrospun nanomaterials are ideal electrode materials in the field of bioelectrochemistry and electroanalytical chemistry because of their unique structures such as high specific surface area, high porosity and three-dimensional reticular structure [17-25]. In particular, the electrospun polymer nanofibers have good biocompatibility and could provide a promising matrix for protein or enzyme immobilization and be used for biosensor fabrication. Therefore, we proposed to fabricate the hemoglobin-collagen-carbon nanotubes (Hb-collagen-CNTs) composite nanofibers by electrospinning to enhance the sensitivity and detection limit of hydrogen peroxide biosensor due to the subtle electronic properties of CNTs, the excellent biocompatibility of collagen as natural polymer and high specific surface area, high porosity and three-dimensional reticular structure of electrospun nanofibers. The morphology of Hb-collagen-CNTs composite nanofibers was characterized by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The determination and analysis of hydrogen peroxide were carried out by cyclic voltammetry and amperometric detection.

2. Experiments

2.1 Materials

Bovine hemoglobin (Hb, M_w 66,000, the isoelectric point $pI \sim 7.4$) was purchased from Sigma Chemical Co. and was used without any additional purification. Collagen I (mol. W_t , $0.8-1 \times 10^5$ Da) was purchased from Sichuan Mingrang Bio-Tech Co. Ltd (China). CNTs (multi-walled carbon nanotubes with bamboo shaped structure) were obtained from Shenzhen Nanotech Port Co., Ltd (Shenzhen, China) and purified in 3 mol L^{-1} nitric acid for 4 h by sonicating prior to use. The hydrogen peroxide (H_2O_2 , 30%, w/w) and 2,2,2-Trifluoroethanol (TFE) were ordered from Beijing Aladdin Regent Co. (Beijing, China). All other chemicals and solvents were of analytical grade and used without further purification. All aqueous solutions were prepared using doubly distilled water.

2.2 Preparation of the Hb-collagen-CNTs composite nanofibers

Prior to coating, the bare glassy carbon electrodes (GC, 3 mm diameter) were carefully polished with emery paper (# 2000), 0.3 μm and 0.05 μm alumina slurry on a woolen cloth, then cleaned in a ultrasonic bath for 10 min and finally thoroughly rinsed with distilled water. CNTs were dispersed into TFE to obtain a homogeneous dispersion (20 $mg \cdot mL^{-1}$) by sonicating for 4 h. Hb (40 $mg \cdot mL^{-1}$) and collagen (30 $mg \cdot mL^{-1}$) were added into the dispersion under stirring for 15 min to get the homogeneous and stable dispersion, which was found to be essentially stable for at least 2 weeks.

For electrospinning, the dispersion was loaded in a 5 mL plastic syringe equipped with a needle of 0.5 mm diameter and then injected through the needle using an infusion pump (TS2-60, Baoding Longer Precision Pump Co. Ltd., China) at an injection rate of 0.8 $mL \cdot h^{-1}$ and 11 kV voltage (HB-F303-1-AC, China). The surface of the electrode was kept facing to the needle tip. The nanofibers were collected on the surface of the electrode which was kept 10 cm away from the needle tip. The obtained Hb-collagen-CNTs composite nanofibers on the bare GC electrode were crosslinked in glutaraldehyde (GA) vapor for 4 h. For comparison, CNTs (20 $mg \cdot mL^{-1}$) and collagen (70 $mg \cdot mL^{-1}$) were dispersed into TFE by sonicating for 15 min to give a homogeneous dispersion and the dispersion of the CNTs and collagen was electrospun under the same electrospinning conditions with Hb-collagen-CNTs nanofibers to form collagen-CNTs nanofibers on the bare GC electrode. The resulting electrodes were kept in 0.10 $mol \cdot L^{-1}$ phosphate buffer solution for at least 1 h prior to the electrochemical measurements. The enzyme electrodes were stored at 4 $^{\circ}C$ in a refrigerator when they were not in use.

2.3 Characterization

Scanning electron microscopy (SEM) (MX2600FE, Camscan Company, UK) was conducted to characterize the morphologies of the electrospun Hb-collagen-CNTs composite nanofibers. All samples were coated with a thin layer of platinum in two 30 s consecutive cycles at 45 mA to reduce charging and produce a conductive surface. Transmission electron microscopy (TEM, FEI Tecnai G2 S-Twin, Philips-FEI, Holland) was used to record the structure of nanofibers and the distribution of the CNTs within nanofibers. UV-vis

absorbance spectroscopy was obtained on a UV-Vis spectrometer 2550 (Shimadzu, Japan). Electrochemical measurements were performed with a computer-controlled electrochemical analyzer (CHI 650C, Austin, TX) at 20 ± 2 °C. A conventional three-electrode cell was used, the modified electrodes as working electrodes, a platinum spiral wire as counter electrode and a saturated calomel electrode (SCE) as reference electrode. The electrolyte was $0.10 \text{ mol} \cdot \text{L}^{-1}$ phosphate buffer solution (pH 7.0), which was bubbled with pure nitrogen gas for more than 30 min before electrochemical measurements to maintain the anaerobic condition. And then nitrogen gas was kept flowing over the solution during the electrochemical measurements. The amperometric test was performed under a potential of -0.36 V (vs. SCE) with stirring. The electrochemical measurement was performed five times with five modified electrodes prepared at one time to ensure the reproducibility.

3. Results and discussion

3.1 Characterization of electrospun Hb-collagen-CNTs nanofibers

The surface morphology of Hb-collagen-CNTs nanofibers is shown in Fig. 1A. The surfaces of Hb-collagen-CNTs nanofibers were smooth without any bead defects. The average diameter of Hb-collagen-CNTs nanofibers was $190 \pm 59 \text{ nm}$, which was much smaller than that of electrospun CNTs-Hb and Hb-collagen microbelts [22,23]. A possible reason is that the addition of CNTs and collagen decreased the viscosity of the composite solution and increased the charge density of the solution which improved the stretching force and the self-repulsion of the jet, and thus made the fiber diameter reduce. However, it was observed that the diameters of fibers were not uniform, which might be due to the aggregation of CNTs in the nanofibers. In addition, the electrospun nanofibers possessed the three-dimensional network structure with larger specific surface area and higher porosity, which is very beneficial to facilitate the direct electron transfer between protein or enzyme and electrode. In order to determine the distribution of CNTs in the nanofibers, TEM image was taken to reveal the internal mesostructure of the Hb-collagen-CNTs nanofibers. As shown in Fig. 1B, the majority of CNTs were randomly immobilized in the nanofibers, while a small amount of CNTs were protruded from the surface of the nanofibers (as indicated by arrows).

Moreover, to examine the activity of the protein in the electrospun nanofibers, UV-vis spectrometer was used to characterize the structure of Hb immobilized in the electrospun composite nanofibers. And the information about the surrounding environment of the heme prosthetic group of Hb was obtained [26]. Fig. 1C shows the UV-vis absorption spectra of the Hb-collagen-CNTs nanofibers, the collagen-CNTs nanofibers and the native Hb. The Soret band of the native Hb was characterized at 410 nm and the Soret band of Hb entrapped in the electrospun nanofibers shifted to 402 nm . This slight shift was probably caused by the interaction between collagen molecules and Hb in electrospun nanofibers.

Here Fig. 1

3.2 Direct electrochemistry of Hb-collagen-CNTs nanofibers

The cyclic voltammograms (CVs) of Hb-collagen-CNTs nanofibers (solid line) modified and collagen-CNTs nanofibers (dashed line) modified electrodes are shown in Fig.

2. The Hb-collagen-CNTs nanofibers modified electrode exhibited a typical CV with a pair of well-defined redox peaks at -0.31 V and -0.42 V (vs. SCE) in a 0.10 mol·L⁻¹ phosphate buffer solution (pH 7.0) under nitrogen saturated at a scan rate of 100 mV·s⁻¹. The formal potential (E^0) defined as the average of anodic peak potential and cathodic peak potential was -0.365V (vs. SCE), which was close to the redox potential of Fe(III)/Fe(II) of the heme group in Hb reported in the literatures [27-29]. It is obvious that the direct electron transfer between the electrode and Hb immobilized in the electrospun nanofibers could be accomplished due to the subtle electronic properties of CNTs and the favorable micro-environment for Hb immobilization provided by collagen with good biocompatibility.

Here Fig. 2

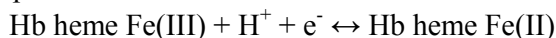
Along with the increase of scan rate in the range from 100 to 900 mV·s⁻¹, the cathodic and anodic peak potentials shifted towards negative and positive directions respectively, while the peak currents increased linearly with the scan rate. It indicated that the electron transfer process between the electrode and the Hb immobilized in the electrospun nanofibers was a surface controlled electrochemical behavior. The apparent heterogeneous electron transfer rate constants (k_s) could be calculated according to Laviron's equations [30], as the peak-to-peak separation (ΔE) was larger than 200 mV:

$$E_{p,c} = E^0 + \frac{RT}{\alpha F} \ln \frac{RTk_s}{\alpha F\nu}; \quad E_{p,a} = E^0 - \frac{RT}{(1-\alpha)F} \ln \frac{RTk_s}{(1-\alpha)F\nu}$$

$$\Delta E_p = E_{p,a} - E_{p,c} = \frac{RT}{\alpha(1-\alpha)F} \left[-\ln \frac{RTk_s}{F\nu} + (1-\alpha) \ln \alpha + \alpha \ln (1-\alpha) \right]$$

where α is the electron transfer coefficient. The value of k_s was evaluated as 5.3±0.13 s⁻¹, which was higher than that of Hb immobilized in Fe₃O₄@Al₂O₃ nanoparticles (4.3 s⁻¹) [31], Ag/Ag₂V₄O₁₁ nanocomposite (2.6 s⁻¹) [32], mesoTiO₂ (1.9±0.15 s⁻¹) [33] and nanocrystalline tin oxide (1.02±0.05 s⁻¹) [34]. The reasons might be as follows. The collagen as a natural polymer has good biocompatibility and could provide a suitable matrix for Hb immobilization. The high porosity of three-dimensional reticular structure of the electrospun nanofibers provide plenty of channels for the electron transfer between Hb and electrode. Moreover, CNTs in the electrospun nanofibers improve the electron conductivity due to its eximious electronic properties.

Fig. 3A shows the CVs of Hb-collagen-CNTs nanofibers modified electrode in 0.10 mol·L⁻¹ phosphate buffer solution with different pH values from 5.0 to 9.0. As shown in Fig. 3A, the formal potential E^0 shifted toward negative when the pH of electrolyte increased from 5.0 to 9.0. The E^0 decreased linearly with the pH of the solution at a slope of -52.9 mV·pH⁻¹, as displayed in Fig. 4B ($R=0.999$). The slope was similar to the Nernstian value of -58 mV·pH⁻¹ at 20 °C, indicating the reversible electron transfer process of one electron and one proton reaction [35]. The reduction scheme of Hb in Hb-collagen-CNTs nanofibers could be expressed as follows:



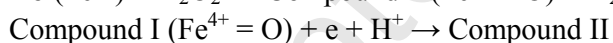
It was observed from Fig. 3B that the peak current (I_p) changed with the pH of the

solution, and the maximum peak current was obtained at pH 7.0. In order to maintain good biological activity of the protein, the phosphate buffer solution with pH=7.0 was chosen for the following experiments.

Here Fig. 3

3.3 H_2O_2 biosensor based on Hb-collagen-CNTs nanofibers

Nowadays, hydrogen peroxide has been extensively used in the environment, food, chemical, pharmaceutical and other fields. However, the existence of H_2O_2 will do harm to the people's health if its concentration exceeds certain limits. Therefore, the accurate and fast measurement of H_2O_2 is of great significance. Hb could be used to determine the concentration of H_2O_2 because of its similar structure to peroxidase with an intrinsic catalytic activity toward peroxide compounds [36]. Electrocatalytic behaviors of the Hb-collagen-CNTs nanofibers modified electrodes were characterized by the cyclic voltammograms. Fig. 4 shows the CVs of the Hb-collagen-CNTs nanofibers modified GC electrodes in 0.10 M phosphate buffer solution (pH 7.0) containing different volumes of H_2O_2 (100, 200, and 400 μ M). The anodic peak current (dashed line) decreased significantly with the increasing concentration of H_2O_2 in the electrolyte, while the reduction peak current increased at about -0.36 V (vs. SCE), suggesting the obvious electrocatalytic behavior of Hb towards the reduction of H_2O_2 . Although the mechanism of catalytic reduction of H_2O_2 by Hb is not yet clear, Serradilla et al. have put forward that the electrocatalytic behavior of H_2O_2 could be interpreted as the following [37]. Firstly, ferric Hb may be oxidated by H_2O_2 forming the intermediate Compound I ($Fe^{4+} = O$). Then the intermediate may be reduced into ferric Hb through a two-electron reaction at the modified electrode at the potential ferric Hb reduction. The process could be explained as follows [23]:



Here Fig. 4

For the sensitive and accurate detection of H_2O_2 , the amperometric response of the collagen-CNTs nanofibers (curve a) and Hb-collagen-CNTs nanofibers (curve b) modified electrodes to the successive additions of H_2O_2 into 0.10 mol·L⁻¹ phosphate buffer solution (pH 7.0) at an applied potential of -0.36V (vs. SCE) was studied by current-time curve (Fig. 5A). When the H_2O_2 was added into the electrolyte, for the Hb-collagen-CNTs nanofibers modified electrodes, the current intensity changed in a very short time and then achieved the stable state after about 10 s, but the current had no change with increasing the H_2O_2 concentration for the collagen-CNTs nanofibers modified electrodes. It suggested that Hb had close structural similarity to the peroxidase with an intrinsic catalytic activity toward the peroxide compounds and the biosensor based on Hb-collagen-CNTs nanofibers possessed high sensitivity to H_2O_2 due to the greater specific surface area and high porosity of the electrospun nanofibers.

Fig. 5B shows the steady-state response of the current in good linear relationship with

the concentration of H_2O_2 ranging from 5.0 μM to 200 μM ($R=0.999$). The linear range of the H_2O_2 biosensors was widened compared with the Hb-collagen nanofibers modified electrode (5.0 to 30 μM) [23]. At a signal to noise ratio of 3, the detection limit of the H_2O_2 biosensors was 0.91 $\mu\text{mol}\cdot\text{L}^{-1}$ (Table 1), which was smaller than the value reported previously (Table 1). It might be due to the three-dimension architecture of the electrospun Hb-collagen-CNTs nanofibers, which is favorable for the effective diffusion of H_2O_2 from the solution to the interface and increased the contact area between Hb and the target. The result also suggests that the Hb-collagen-CNTs nanofibers modified electrodes have promising application for the determination of H_2O_2 . In addition, the reproducibility of the biosensor based on Hb-collagen-CNTs nanofibers expressed as the R.S.D. was 3.76% ($n=5$) when the concentration of H_2O_2 was 100 μM . The biosensor retained 90% response to H_2O_2 after stored in 0.10 M phosphate buffer solution (pH 7.0) at 4°C for 3 weeks.

As seen from Fig. 5B, there was a plateau when the concentration of H_2O_2 increased to 200 μM , exhibiting the Michaelis-Menten kinetic behavior. The apparent Michaelis-Menten constant (K_m^{app}) calculated from Lineweaver-Burk equation [40] was 32.6 μM , which was much smaller than that reported previously [23, 41-45]. These results indicated that the biosensor based on the Hb-collagen-CNTs composite nanofibers had a higher affinity to H_2O_2 .

Here Fig. 5

It demonstrates that the proposed H_2O_2 biosensors based on the Hb-collagen-CNTs composite nanofibers possess the excellent performance. The main reason is the greater specific surface area and higher porosity of the three-dimensional reticular structure of the electrospun Hb-collagen-CNTs composite nanofibers modified electrode, which provides larger contact areas and more channels for the electron transfer and the diffusion of H_2O_2 . Additionally, the subtle electronic properties of CNTs speed up the electron transfer process from activation center of Hb to the electrode surface. Furthermore, the good biocompatibility of collagen is beneficial for the Hb immobilization on Hb-collagen-CNTs composite nanofibers modified electrode surface. Therefore, the biosensor based on the electrospun Hb-collagen-CNTs composite nanofibers possesses the advantages of high sensitivity, low detection limit and good reproducibility.

4. Conclusions

The electrospun Hb-collagen-CNTs composite nanofibers modified electrode with three-dimensional porous architecture have outstanding conductivity and good biocompatibility. The Hb-collagen-CNTs nanofibers modified electrode could accomplish the fast direct electron transfer between Hb and the electrode. The formal potential $E^{0'}$ shifted linearly with the pH of the solution with a slope of -52.9 $\text{mV}\cdot\text{pH}^{-1}$. The electrospun Hb-collagen-CNTs nanofibers modified electrode might be used to measure the concentration of H_2O_2 with high sensitive, low detection limit, acceptable reproducibility toward H_2O_2 . It is expected that this study will be useful for the fabrication of new biosensors based on the electrospun nanofibers.

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1 **Figure Captions**

2 **Fig. 1** (A) SEM and (B) TEM images of the electrospun Hb-collagen-CNTs composite
3 nanofibers. (C) UV-vis absorption spectra of Hb-collagen-CNTs nanofibers, collagen-CNTs
4 nanofibers and native Hb.

5
6 **Fig. 2** Cyclic voltammograms (CVs) of the collagen-CNTs nanofibers (dashed line) and
7 Hb-collagen-CNTs nanofibers (solid line) modified electrodes in 0.10 mol·L⁻¹ phosphate
8 buffer at scan rate of 100 mV·s⁻¹.

9
10 **Fig. 3** (A) CVs of the Hb-collagen-CNTs nanofibers modified electrode in the phosphate
11 buffer solution with various pH values. (B) The plot of the formal potential and the peak
12 current vs. pH value. Data are shown as mean ± SD (n=5).

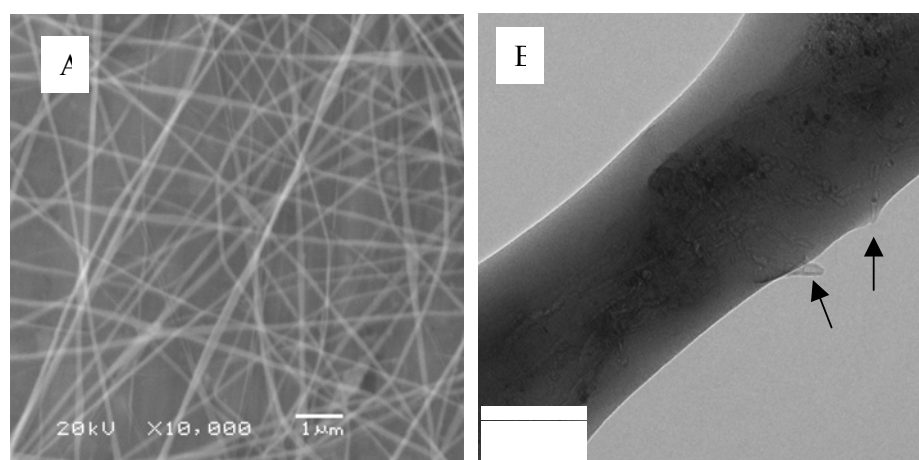
13
14 **Fig. 4** CVs of the Hb-collagen-CNTs composite nanofibers modified electrode in 0.10
15 mol·L⁻¹ phosphate buffer solution (pH 7.0) with different concentrations of H₂O₂ at a scan
16 rate of 100 mV·s⁻¹.

17
18 **Fig. 5** (A) Amperometric responses of the collagen-CNTs nanofibers (curve a) and
19 Hb-collagen-CNTs composite nanofibers (curve b) modified electrode in the stirred 0.10
20 mol·L⁻¹ phosphate buffer solutions (pH 7.0) with successive additions of H₂O₂. Applied
21 potential: -0.36 V vs. SCE; (B) The calibration curve for the concentrations of H₂O₂, inset
22 shows the corresponding Lineweaver-Burk plot. Data are shown as mean ± SD (n=5).

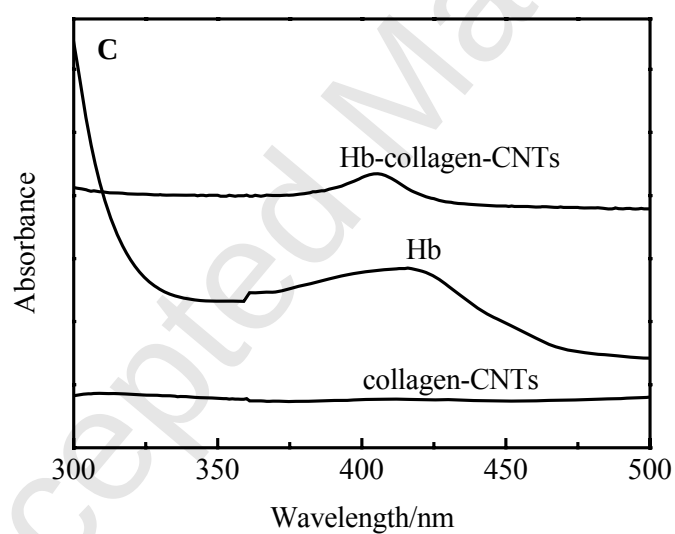
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1 **Fig. 1 – Li *et al.***

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1 **Fig. 2 – Li *et al.***

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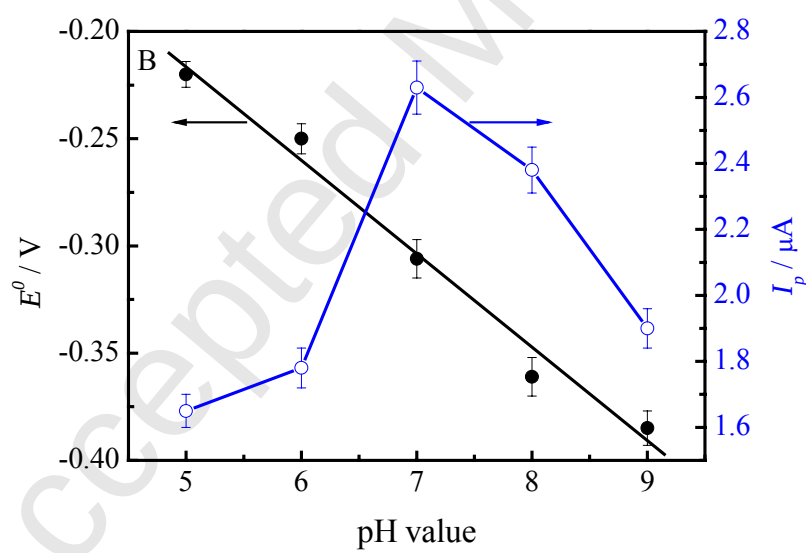
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1 Fig. 3 –Li *et al.*

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1 **Fig. 4 –Li *et al.***

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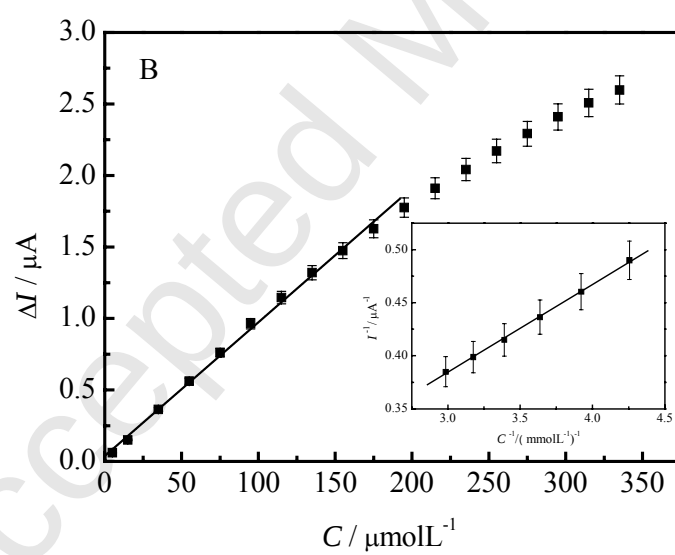
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1 Fig. 5 –Li *et al.*

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Table.1 –Li et al.

Table 1. Comparison of the properties between the H₂O₂ biosensors based on the direct electron transfer of the Hb immobilized on different modified electrodes.

Different H ₂ O ₂ sensors	Liner range (μM)	Detection limit (μM)	Ref
Hb/SWNT	6 to 600	1.2	[22]
Hb/CMC-TiO ₂ -NT	4 to 64	4.637	[25]
Hb/mesoTiO ₂	2 to 27.5	1	[30]
Hb/NGC-SF	600 to 1700	100	[38]
Hb/P123-NGP	10-150	8.24	[39]
Hb-collagen-CNT nanofibers	5 to 200	0.91	Present study

Note: CMC-TiO₂-NT: Carboxymethyl cellulose titaniumoxide nanotubes; mesoTiO₂: Highly ordered mesoporous titanium oxide; NGC-SF: Nanostructured gold colloid-silk fibroin; P123-NGP: Pluronic P123-nanographene platelets composite