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A novel amperometric hydrogen peroxide biosensor based on electrospun Hb–collagen composite

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ABSTRACT

In this paper, the hemoglobin (Hb)–collagen microbelt modified electrode with three-dimensional configuration was fabricated via the electrospinning method. Direct electron transfer of the Hb immobilized into the electrospun collagen microbelts was greatly facilitated. The apparent heterogeneous electron transfer rate constant (k_s) was calculated to be 270.6 s^{-1}. The electrospun Hb–collagen microbelt modified electrode showed an excellent bioelectrocatalytic activity toward the reduction of H_2O_2. The amperometric response of the biosensor varied linearly with the H_2O_2 concentration ranging from 5×10^{-6} mol L^{-1} to 30×10^{-6} mol L^{-1}, with a detection limit of 0.37×10^{-6} mol L^{-1} (signal-to-noise ratio of 3). The apparent Michaelis–Menten constant (K_m^{app}) was 77.7 μmol L^{-1}. The established biosensor exhibited fast amperometric response, high sensitivity, good reproducibility and stability.

1. Introduction

Considerable interests have been aroused for the electrochemical biosensor research in recent years [1–5] due to its superiorities, such as high sensitivity, nice selectivity, fast response, low cost, continuous on-line detection in complex system and convenient electroanalysis in a compact form to facilitate analysis [6]. At present, the amperometric hydrogen peroxide (H_2O_2) biosensors based on biomolecule-immobilized (e.g. protein and enzyme) is promising for the fabrication of simple and low-cost enzyme sensors. Because of close structural similarity to the peroxidase, hemoglobin (Hb), an iron-containing oxygen-transport metalloprotein in the mammals' red blood cells, can be used as a substitute of the peroxidase with an intrinsic catalytic activity toward peroxide compounds [7]. However, the rate of direct electron transfer of Hb is rather slow when it is immobilized on the bare electrode [8]. To overcome this problem, great efforts have been made to facilitate the direct electron transfer of protein or enzyme by immobilizing them into the suitable matrix such as layer-by-layer assembly film [9,10], ionic liquid [11,12], lipid [13], conducting polymer [14], hydrogel polymer [15,16], biopolymer [17,18] and nanomaterials [19–25]. Among these means, nanomaterials used as substrates for immobilizing redox enzymes or proteins have attracted considerable attention recently due to its superior structural stability and size effect.

Electrospinning is a feasible technique to produce continuous polymer-based fibres with diameters ranging from nanometer to micrometer scale and has shown potential in the synthesis of nanomaterials [26–30]. When a high voltage is applied, the increasing electric charge will cause the pendent droplet during the process of electrospinning at the nozzle, which deforms from hemispherical to conical in shape and is referred as "Taylor cone". Once electrostatic force overcomes the surface tension, a jet of solution would eject from droplet and undergo a process of stretching, splitting and whipping in the air and finally form into polymer fibre when the solvent evaporates from the jet. Electrospun nanofibres can be applied in many fields such as biomedical fields [26–28], sensors [31], reinforced composite materials [32] and filter materials [33] because the nanofibres produced via electrospinning own unique structures: nanoscale in diameter and high specific surface area of single fibre, high porosity and three-dimensional reticular structure.

Collagen is one of the main classes of structural natural extracellular matrix proteins, and has been commonly used as a biomaterial in a variety of tissue applications due to its excellent biocompatibility [34]. Therefore, in the present study, the Hb–collagen microbelt modified electrode was prepared via the electrospinning method and its morphology was investigated by scanning

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electron microscope (SEM). Direct electron transfer of the electrospun Hb–collagen microbelt and its amperometric response toward H_2O_2 were further investigated.

2. Experiments

2.1. Materials

Collagen I (mol. wt, 0.8–1 × 10^5 Da) was purchased from Sichuan Mingrang Bio-Tech Co. Ltd (China). Hemoglobin (Hb, from bovine blood, M_W 66,000, lyophilized powder, the isoelectric point p$I \sim 7.4$) was purchased from Sigma Chemical Co. and was used without any additional purification. The hydrogen peroxide (H_2O_2, 30% (w/w)) and 1,1,1,3,3,3 hexafluoro-2-propanol (HFP) were purchased from Beijing Chemical Company (Beijing, China). The dilute solution of H_2O_2 was prepared daily. All other chemicals and solvents were of analytical grade and used without further purification. All aqueous solutions were prepared using doubly distilled water.

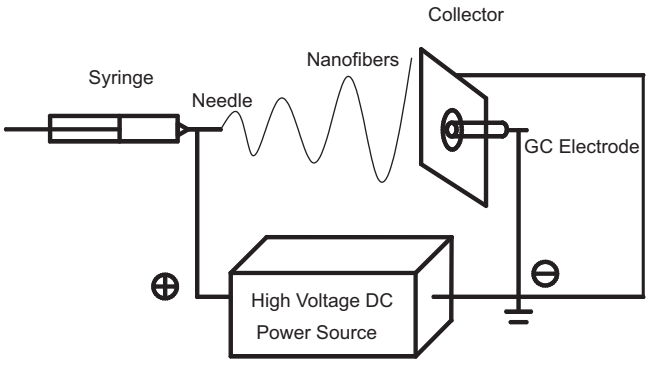
2.2. Preparation of biosensor

Prior to coating, the bare glassy carbon (GC, 3 mm-diameter) was firstly polished with emery paper (# 2000), 0.3 and 0.05 μm alumina slurry on a woolen cloth, then cleaned under ultrasonic bath for 10 min and finally thoroughly rinsed with doubly distilled water.

For the preparation of the electrospun Hb–collagen/GC modified electrodes, the Hb–collagen composites were prepared firstly by dissolving collagen (10 mg mL^{-1}) and Hb (175 mg mL^{-1}) in HFP, respectively. The composite solution was transferred into a 5 mL plastic syringe fitted with a stainless-steel blunt needle of 0.5 mm in diameter at an injection rate of 0.6 mL h^{-1} using an infusion pump (TS2-60, Baoding Longer Precision Pump Co. Ltd., China) between the fresh bare GC electrode used as collector and the needle tip of 12 cm under a driving voltage of 11 kV by high voltage power supply (HB-F303-1-AC, China), as shown in Scheme 1. The resulting electrospun Hb–collagen onto the surface of bare GC electrode was further crosslinked in glutaraldehyde vapor. Then the modified electrode was stored at 4 °C in a refrigerator when not in use and was immersed into 0.10 M phosphate buffer for more than 2 h prior to the electrochemical measurements in order to wet the modified electrode.

2.3. Apparatus and characterization

Morphological examinations of electrospun collagen fibres and Hb–collagen microbelts were carried out by scanning electron microscope (SEM) (MX2600FE, Camscan Company, UK). All samples were coated with a thin layer of gold in two 30 s consecutive cycles at 45 mA to reduce charging and produce a conductive surface. The diameters of obtained microbelts were analyzed using software of Image J. The electrochemical data were obtained with a computer-controlled electrochemical analyzer (CHI 650 C, CHI, Austin, TX) in a two-compartment and three-electrode cell. The prepared electrospun Hb–collagen/GC electrodes were used as working electrodes, a platinum spiral wire as the counter electrode and a saturated calomel electrode (SCE) as the reference electrode. A 0.10 mol L^{-1} phosphate buffer (pH 7.0) was used as the supporting electrolyte. For the experiments conducted under anaerobic condition, prior to electrochemical measurements, the electrolyte was bubbled with pure nitrogen gas for more than 30 min, and nitrogen gas was kept flowing over the solution during all electrochemical measurements. All electrochemical measurements were performed at least three times at room temperature (20 ± 2 °C). In addition, the electrochemical measurement was repeated five times with five enzyme electrodes prepared at one time to ensure the reproducibility.



Scheme 1. Schematic illustration of electrospinning apparatus applied to produce the collagen nanofibres and Hb–collagen microbelt modified GC electrode.

3. Results and discussion

3.1. Characterization of electrospun collagen and Hb–collagen composite

Fig. 1 is the typical SEM images showing the morphologies of electrospun collagen and Hb–collagen composite. As can be seen from Fig. 1, uniform and highly smooth electrospun collagen (Fig. 1A) and Hb–collagen (Fig. 1B) were formed without the bead defects for all the electrospun samples. Compared with electrospun collagen nanofibres (Fig. 1A), the electrospun Hb–collagen exhibited the microbelt-shaped structure (marked by an arrow in Fig. 1B) with an average width of about 2.3 μm and average thickness of about 450 nm. The three-dimensional configuration of the electrospun Hb–collagen consisted of single separated microbelts shows no aggregation at all because of the strong attractive interactions of these hydrophobic surfaces among each other, which is very beneficial to facilitate the direct electron transfer of enzyme or protein.

3.2. Direct electrochemistry of electrospun Hb–collagen modified electrode

The direct electrochemistry behavior of electrospun Hb–collagen modified electrode was studied as shown in Fig. 2. It can be seen in cyclic voltammograms (CVs) (solid line in Fig. 2) that a pair of well-defined redox peaks was observed at the electrospun Hb–collagen/GC electrode in a N_2-saturated 0.10 mol L^{-1} phosphate buffer solution of pH 7.0 at a scan rate of 200 mV s^{-1} compared to the electrospun collagen/GC electrode (dot line in Fig. 2). Its anodic peak potential and cathodic peak potential were located at −0.29 V and −0.39 V (vs. SCE), respectively, which was consistent with the reported potential values for the Fe(III)/Fe(II) redox center of the heme group in the Hb [35–37]. These results suggested that direct electron transfer of the Hb molecules entrapped in the electrospun collagen microbelts was enhanced. In addition, it can be easily found from Figs. 1S and 2S that the anodic and cathodic peak currents obtained at the electrospun Hb–collagen/GC electrodes were significantly affected by electrospun time and crosslink time of Hb–collagen composite, which could be explained as follows. On the one hand, the thickness of electrospun Hb–collagen microbelts increased with the prolonged electrospinning time, which resulted in the increased charge transfer resistance because collagen itself is not conductive. On the other hand, the anodic and cathodic peak currents obtained at

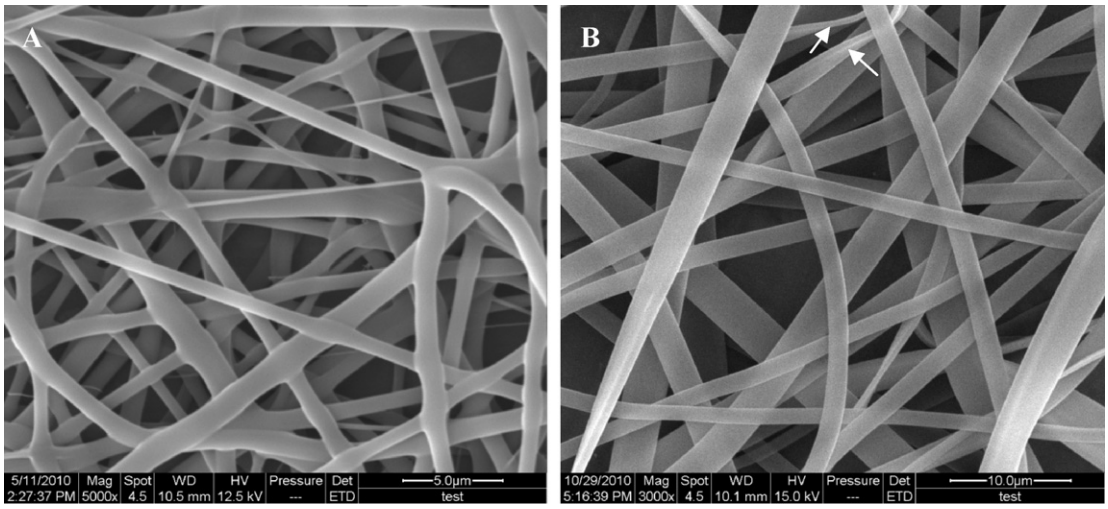


Fig. 1. SEM images of electrospun collagen nanofibres (A) and Hb–collagen microbelts (B).

the electrospun Hb–collagen/GC electrodes decreased sharply as the crosslink time increased, because the elevated intermolecular force by crosslinking could result in the denaturation of proteins and unfavorable orientations at electrodes. Therefore, the optimal electrospun time and crosslink time are 10 min and 5 h, respectively, and were selected for the following experiments.

Fig. 3A shows the cyclic voltammograms of the electrospun Hb–collagen/GC electrodes in 0.1 mol L^{-1} phosphate buffer solution of pH 7.0 at different scan rates from 100 mV s^{-1} to 900 mV s^{-1}. The peak currents increased and the cathodic and anodic peak potentials exhibited a small shift along with the increase of scan rate. At the same time, the cathodic and anodic peak currents increased linearly with the scan rate (not $v^{1/2}$), as shown in Fig. 3B. All these results indicated that the Hb immobilized into electrospun collagen microbelts underwent a surface controlled and quasi-reversible electrochemical reaction process.

When the peak-to-peak separation (ΔE) was larger than 200 mV, the apparent heterogeneous electron transfer rate constants (k_s) would be easily calculated with the help of Laviron's

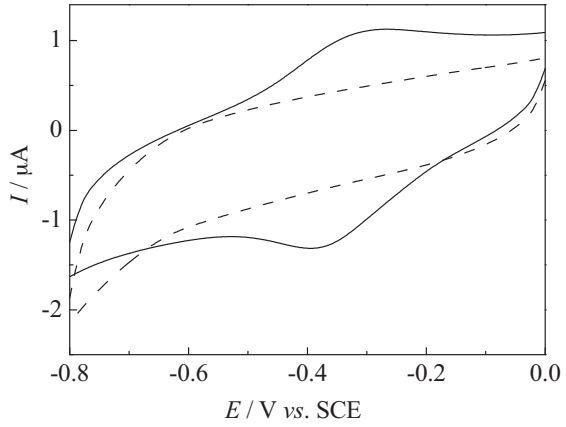


Fig. 2. Cyclic voltammograms (CVs) obtained at electrospun collagen nanofibres/GC (dashed line) and Hb–collagen/GC (solid line) modified electrodes in 0.10 mol L^{-1} N_2-saturated phosphate buffer. Scan rate, 200 mV s^{-1}.

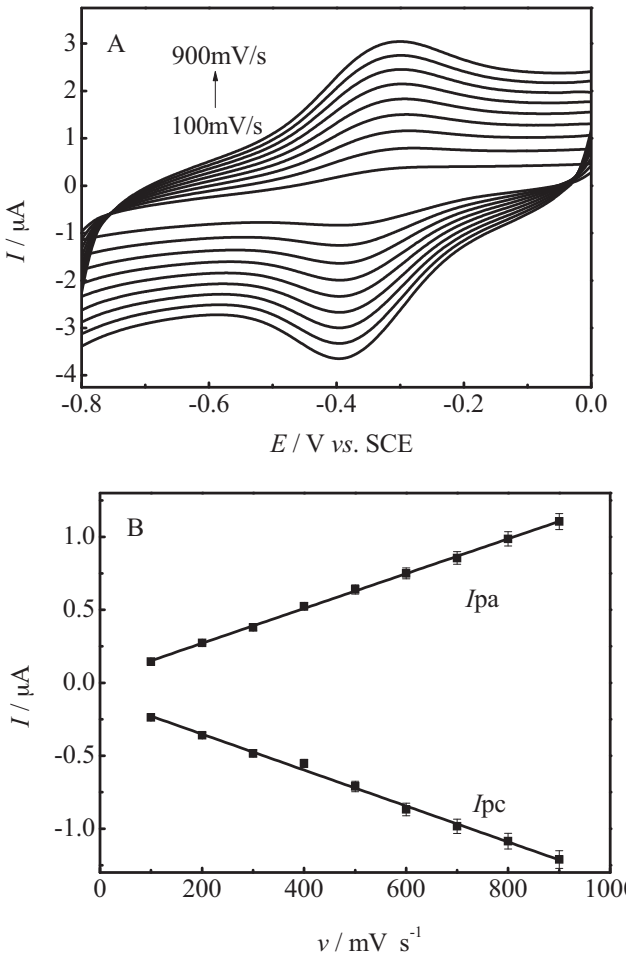


Fig. 3. (A) CVs obtained at the electrospun Hb–collagen/GC electrode in 0.10 mol L^{-1} N_2-saturated phosphate buffer solution at 100, 200, 300, 400, 500, 600, 700, 800 and 900 mV s^{-1} (from inner to outer). (B) Plot of cathodic and anodic peak currents vs. scan rate. Data are shown as mean ± SD (n = 5).

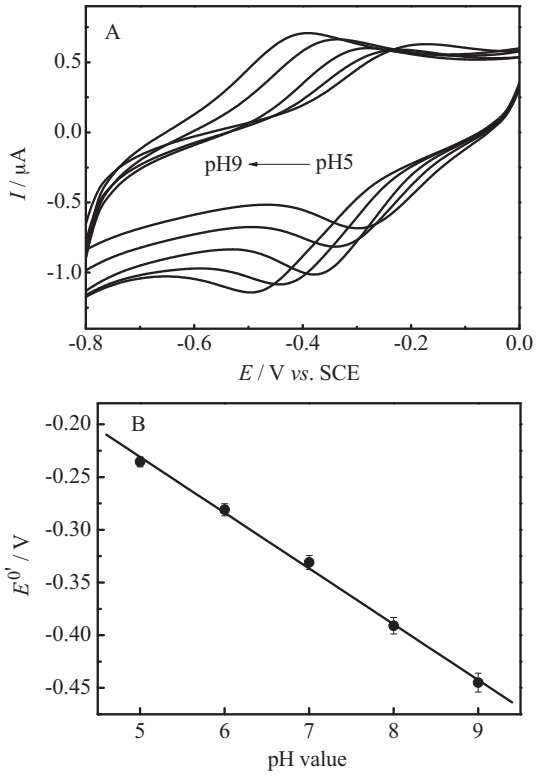


Fig. 4. (A) CVs of the electrospun Hb–collagen/GC electrode in phosphate buffer solution with different pH values. (B) Dependence of pH value on $E^{0'}$ of electrospun Hb–collagen/GC electrode in 0.10 mol L^{-1} phosphate buffer. Scan rate: 200 mV s^{-1}. Data are shown as mean ± SD (n = 5).

equations [38] as follows:

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

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

where α is the electron transfer coefficient and k_s is the apparent heterogeneous electron transfer rate constants which can be calculated according to ΔE_p versus $\ln\nu$. The value of k_s was calculated to be 270.6 s^{-1}, which is much higher than that of Hb immobilized in Pluronic P123-NGPs nanocomposite (48.51 s^{-1}) [39], $Bi_4Ti_3O_{12}$ (20.0 ± 3.8 s^{-1}) [20], Fe_3O_4@Al_2O_3 nanoparticles (4.3 s^{-1}) [25], and Ag/$Ag_2V_4O_{11}$ nanocomposite (2.6 s^{-1}) [40]. The k_s value directly shows that electrospun Hb–collagen microbelts enhanced the electron transfer rate between Hb and electrode, and it can be explained as follows. Firstly, the electrospun collagen nanofibres would provide an appropriate matrix for protein or enzyme immobilization because of their satisfying biocompatibility. Secondly, the advantages of electrospun Hb–collagen microbelts possessed the three-dimensional reticular structure, high specific surface area, interconnect pores and high porosity, which would be favorable for the electron transfer of proteins away from electrode.

The effect of the pH value of the electrolyte on the electrochemical behavior of the electrospun Hb–collagen/GC electrodes has been also investigated as shown in Fig. 4A. The result showed that the increase in pH value of the phosphate buffers from 5.0 to 8.0 led to a negative shift of the anodic and cathodic peak potentials for the electrospun Hb–collagen/GC modified electrodes. Moreover, it can be seen from Fig. 4B that the $E^{0'}$ exhibited linear relationship with

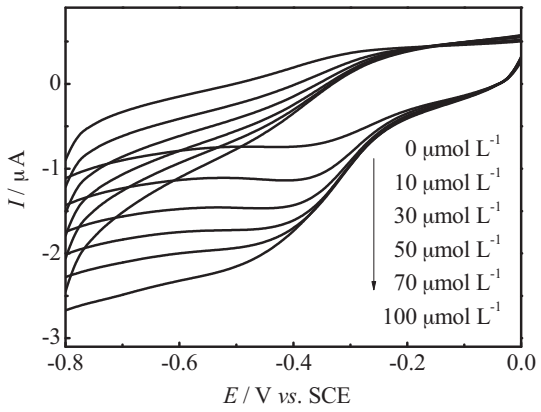


Fig. 5. CVs of the electrospun Hb–collagen/GC electrode in 0.10 mol L^{-1} pH 7.0 phosphate buffer solution containing different contents of H_2O_2. Scan rate, 200 mV s^{-1}.

the pH value in the range of 5.0–9.0 and the slope of −52.9 mV pH^{-1} (the correlative coefficient is 0.999) is close to the theoretical value of −58 mV pH^{-1} at 20 °C for one electron and one proton reaction in the electron transfer process [41]. The electrochemical reduction scheme of Hb can be simply expressed as follows:

$$\text{HbhemeFe(III)} + H^+ + e^- \rightarrow \text{HbhemeFe(II)}$$

3.3. H_2O_2 biosensor based on electrospun Hb–collagen

The detection of hydrogen peroxide (H_2O_2) is of great importance since it is an essential compound in environmental, industrial, chemical, food, pharmaceutical and biological fields. Titrimetry, spectrometry, chemiluminescence and electrochemical measurement are the main methods used to detect hydrogen peroxide. Among them, the amperometric enzyme-based biosensors coupled with the intrinsic selectivity and sensitivity of enzymatic reactions for the determination of H_2O_2 has attracted considerable attention because of their good sensitivity, high selectivity and convenience [42]. Fig. 5 shows the cyclic voltammograms obtained at the electrospun Hb–collagen modified GC electrode in the presence of different volumes of H_2O_2. When the H_2O_2 was added into the phosphate buffer, the cathodic peak current at about −0.35 V (vs. SCE) increased dramatically with the decrease of the anodic peak (dashed line) at the electrospun Hb–collagen/GC electrodes, and the reduction peak current increased with an increase of H_2O_2 concentration in phosphate buffer, which indicated a typical electrocatalytic activity of the electrospun Hb–collagen toward the reduction of H_2O_2. The mechanism can be expressed as the following equations [43]:

$$\text{Hb}(Fe^{3+}) + H_2O_2 \rightarrow \text{CompoundI}(Fe^{4+}{=}O) + H_2O$$

$$\text{CompoundI}(Fe^{4+}{=}O) + e + H^+ \rightarrow \text{CompoundII}$$

$$\text{CompoundII} + e + H^+ \rightarrow \text{Hb}(Fe^{3+}) + H_2O$$

The amperometric response of the electrospun Hb–collagen/GC modified electrodes upon the successive additions of H_2O_2 to 0.10 M pH 7.0 PBS at an applied potential of −0.38 V (vs. SCE) is shown in Fig. 6. As can be seen, the steady-state response current increased as the H_2O_2 concentration increased. Moreover, the current response shows the linear relationship with the concentration of H_2O_2 ranging from 5.0×10^{-6} mol L^{-1} to 30×10^{-6} mol L^{-1} (the correlative coefficient is 0.999), and the detection limit was 0.37 μmol L^{-1} based on a signal-to-noise ratio of 3 (left side inset of Fig. 6). The limitation value was smaller than the corresponding values reported with other protein modified electrodes (Table 1),

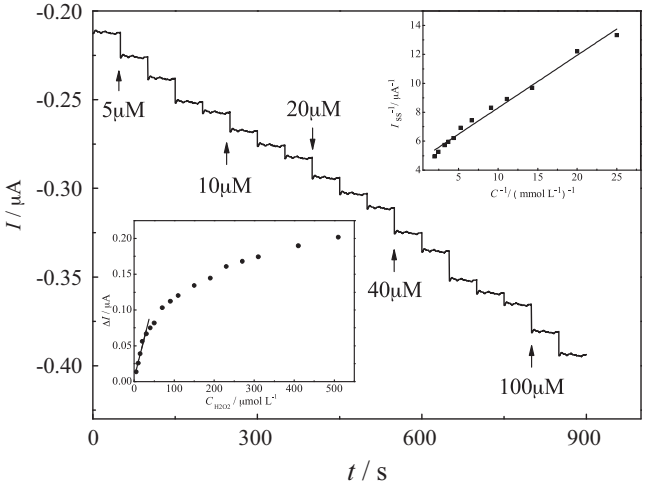


Fig. 6. Amperometric responses of the electrospun Hb–collagen/GC electrode in stirred solutions to the successive additions of H_2O_2. Applied potential: −0.38 V vs. SCE; supporting electrolyte: 0.10 mol L^{-1} pH 7.0 phosphate buffer solution. Inset shows the calibration curve of catalytic peak current vs. the concentrations of H_2O_2 (left side) and corresponding Lineweaver–Burk plot (top right). Data are shown as mean ± SD (n = 5).

implying that Hb–collagen modified electrode is of higher affinity to H_2O_2. The sensitivity of the present biosensor was found to be 31 nA(μmol L^{-1})$^{-1}$ cm^{-2}, which was higher than that of biosensors based on heme proteins into {collagen/Mb}$_8$ layer-by-layer films (1.606 nA(μmol L^{-1})$^{-1}$ cm^{-2}) [48], and might due to the three-dimension architecture of electrospun Hb–collagen microbelts on electrode surface. The reproducibility of the amperometric biosensor expressed in terms of relative standard deviation was 2.87% (n = 5) for a concentration of 20 μmol L^{-1} H_2O_2. When the H_2O_2 biosensor was stored in a 0.10 M PBS (pH 7.0) at 4 °C after three weeks, the biosensor retained over 90% response of its initial sensitivity to the reduction of H_2O_2, demonstrating a good long-term stability.

On the other hand, a plateau was also observed (top right inset in Fig. 6) when the concentration of H_2O_2 was higher than 30 μmol L^{-1}, exhibiting the characteristic of the Michaelis–Menten kinetic mechanism. The apparent Michaelis–Menten constant (K_m^{app}) can be easily calculated according to the Lineweaver–Burk form of the Michaelis–Menten equation [49] as follows: $1/I_{ss} = 1/I_{max} + K_m^{app}/I_{max}c$. Here I_{ss} obtained from amperometric experiments is the steady-state current after the addition of a substrate, I_{max} is the maximum current under saturated substrate condition, and c is the concentration of the substrate. Therefore, the value of K_m^{app} was calculated to be 77.7 μmol L^{-1}, which was much smaller than that in the previous reports of 176.33 μmol L^{-1} [23], 0.12 mmol L^{-1} [25], 0.25 ± 0.03 mmol L^{-1} [13], and so on [50–52]. More remarkably, the K_m^{app} value was lower than that of the biosensor based on electrospun Hb microbelts (93 μM) [46], which could be due to the fact that the collagen provides a proper matrix for protein immobilization and biosensor fabrication due to its outstanding biocompatibility [48]. These results showed that the electrospun Hb–collagen retained its bioactivity and possessed a high biological affinity to H_2O_2.

Table 1
Linear range and detection limit for different H_2O_2 biosensors based on the direct electron transfer of Hb.

Different H_2O_2 sensors	Liner range (mM)	Detection limit (μM)	Reference
Hb/MWNT/GC	0.006–6	1.2	[44]
Hb/P123	0.001–0.5	0.5	[45]
Hb microbelts/GC	0.01–0.23	0.61	[46]
Hb–MWNT microbelts/GC	12–108	2.41	[47]
Hb–collagen microbelts/GC	0.005–0.03	0.37	This report

4. Conclusions

In the present paper, the Hb–collagen microbelt modified electrode with the three-dimensional configuration was prepared by the electrospinning. Direct electron transfer of the Hb immobilized in electrospun collagen microbelts was easily achieved. The formal potential $E^{0'}$ of the heme Fe(III)/Fe(II) redox couple in Hb varied linearly against the change of pH from 5.0 to 9.0 with a slope of −52.9 mV pH^{-1}. The hydrogen peroxide biosensor based on electrospun Hb–collagen composite microbelts showed a fast amperometric response, high sensitivity, good reproducibility and stability. It is believed that these properties were related to the unique structure of the electrospun Hb–collagen composite microbelts and were very useful for the future development of the new bioelectronic nanodevices such as electrochemical biosensors and enzyme-based biofuel cells.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.colsurfb.2011.03.032.

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