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ORIGINAL PAPER

Acerate ZnO whiskers and sodium alginate films: preparation and application in bioelectrochemistry of hemoglobin

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Abstract A novel biocompatible acerate ZnO whiskers (AZW) has been prepared. We explored AZW and sodium alginate for the construction of electrochemical biosensors. The composition, morphology, and size were studied by scanning electron microscopy. UV–vis spectra revealed that hemoglobin (Hb) adsorbed in the acerate ZnO whiskers and sodium alginate retained its native structure. The amperometric response was measured as a function of H_2O_2 concentration at a fixed potential of −0.25 V in phosphate-buffered saline (pH 7.0). The electrochemical parameters of Hb in acerate ZnO whiskers and sodium alginate were calculated with the results of the electron transfer coefficient (α) and the apparent heterogeneous electron transfer rate constant (k_s) as 0.5 and 2.5 s^{-1}, respectively, indicating good facilitation of the electron transfer between Hb and the modified electrode, which may result from the unique nanostructures and larger surface area of acerate ZnO whiskers. The hydrogen peroxide biosensor showed a fast response of <5 s of linear range 2.1 μM–4.8 mM, with a detection of 0.7 μM (S/N=3). The apparent Michaelis–Menten constant K_m^{app} is 0.8 mM. The biosensor possesses high sensitivity, good reproducibility, and long-term stability.

Keywords Biosensor · Direct electron transfer · Acerate ZnO whiskers · Sodium alginate · Hemoglobin

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Introduction

Direct electron transfer of redox proteins not only provides a model for the study of electron transport of proteins in biological system, which is important to understand the material metabolism and energy transformation in the life process, but also establishes a foundation for fabricating third generation of electrochemical biosensors [1]. Hemoglobin (Hb) is one of the important redox proteins containing four electroactive hemes which have commonly been employed to construct H_2O_2 biosensors because of their commercial availability and peroxidase activity [2]. However, as their redox centers are usually deeply buried within the protein molecules, it is difficult for the electron to direct transfer between proteins and electrode [3]. It is well known that good biocompatibility, strong stability, and high sensitivity are of key importance for electrochemical biosensors to offer effective electron transfer channels between redox-active enzymes and electrodes [4]. Therefore, many nanomaterials, such as nanoporous ZrO_2 [5], multiwall carbon nanotubes and gold colloidal nanoparticles [6], poly(ε-caprolactone) [7], mesoporous carbon [8], mesoporous Al_2O_3 [9], and magnetic chitosan microspheres [10], have been studied as platforms for enzyme immobilization in Hb biosensors.

Metal oxides are known to have the unique ability to promote faster electron transfer kinetics between the electrode and the active site of the desired enzyme [11–13]. Due to the high surface area, high catalytic efficiency, non-toxicity, chemical stability, strong adsorption ability, and high electron communication features [14–18], ZnO exhibits a great potential for the fabrication of efficient chemical and biosensors. Acerate ZnO whiskers with their unique properties could provide a desirable microenvironment for the immobilization of enzymes while retaining

their biological activity, thus leading to an expanded use of these materials for the construction of electrochemical biosensors with enhanced analytical performance. Proteins in acerate ZnO whisker films generally retain enzymes' native structures without modification of their heme Fe(III)/(II) electroactive group. Acerate ZnO whiskers could enhance the direct electron transfer between the enzyme's active sites and the electrode. In order to achieve the best catalytic performance of the enzyme, sodium alginate (SA) has been successfully used as a coating material for preparing robust enzyme biosensors, which are known to be naturally biodegradable polymers with unique properties, enabling them to be used as a matrix for the entrapment of enzyme [19, 20].

In this work, acerate ZnO whiskers was first introduced into the sodium alginate matrix and then electrodeposited onto the electrode so as to provide a larger surface area for anchoring Hb. The obtained biosensor showed good electrocatalytic activity, high sensitivity, good repeatability, long-term stability, and low cost.

Experimental

Reagent and materials

Hb (bovine blood) was obtained from Sigma (China). Phosphate-buffered saline (PBS, 0.1 M) containing 0.1 M KCl with various pH values were prepared by mixing the stock solutions of K_2HPO_4 and KH_2PO_4 and adjusted by 0.1 M KOH and 0.1 M H_3PO_4 solution. Hydrogen peroxide (30%, *w*/*v*, solution) were purchased from Chemical Reagent Company (Chongqing, China), and the concentration of the more diluted hydrogen peroxide solutions prepared from 30% hydrogen peroxide was determined by titration with potassium permanganate. Absolute ethanol, sulfuric acid (H_2SO_4, 98%), zinc oxide (ZnO), and sodium hydroxide (NaOH) were purchased from Chongqing Chemical Reagent Co. All other reagents were of analytical reagent grade and used as received. Doubly distilled water was used in this experiment throughout. Acerate ZnO whiskers were prepared as described in the literature [21], which was prepared by hydrothermal method. A transparent solution saturated with $Zn(OH)_4^{2-}$ was formed by dissolving commercial ZnO powder in 5 M NaOH solution. Then, the $Zn(OH)_4^{2-}$ saturated solution was loaded into distilled water under magnetic stirring for 0.5 h (the volume ratio of $Zn(OH)_4^{2-}$ and H_2O is 2:25). The diluted $Zn(OH)_4^{2-}$ solution was transferred into a Teflon-lined autoclave and maintained at 90 °C for 10 h in an oven. The precipitate was collected by centrifugation, washed with distilled water until neutral, and then dried at 65°°C for 48 h.

Instrumentation

Electrochemical measurements were carried out on CHI 660A electrochemical workstation (CH Instruments, Chenhua Co., Shanghai, China). Fourier transform infrared (FTIR) spectra were obtained using a Nicolet FTIR-170SX Fourier transform infrared spectrometer (Madison, WI, USA). The morphology of acerate ZnO whiskers was investigated by scanning electron microscopy (SEM, HITACHI, S-3000N, Japan).

Preparation of Hb-modified with AZW–SA composite

AZW (2 mg/mL) suspension was obtained by adding 10 mg of as-prepared AZW into 5 mL PBS (pH 7.0) with the aid of ultrasonication. Then, 2 mL AZW suspension was mixed with 2 mL 5 mg/mL Hb solution (pH 7.0) and 1 mL 1 mg/mL SA solution. The resulting Hb/AZW–SA composite was kept at 4 °C and used for further testing.

Preparation of the Hb/AZW–SA-modified electrode

A bulk gold disk electrode (φ=4 mm) was polished to a mirror-like surface with 0.3- and 0.05-μm alumina slurry on microcloth pads. The electrodes were successively sonicated in H_2SO_4 solution (0.5 M), ethanol and doubly distilled water, and then allowed to dry. Hb/AZW–SA/Au electrode was achieved by cycling the potential between −1.1 and +1.2 V for 20 consecutive cycles in the mixed solution. The modified electrode (Hb/AZW–SA/Au) was stored in a refrigerator (4 °C) until further study.

Results and discussion

Characterization of acerate ZnO whiskers

The SEM images of acerate ZnO whiskers are shown in Fig. 1. These acerate ZnO whiskers are composed of uniform nanorods. The diameter of the nanorods is about 4–5 μm.

Spectroscopic analysis

UV–vis spectroscopy is a useful tool for monitoring the possible change of the Soret adsorption band in the heme group region. Figure 2 shows the UV–vis spectra of Hb (a) and Hb/AZW-SA (b). The Soret absorption band of Hb entrapped in AZW–SA locates at about 407 nm (curve b), which is close to that of natural Hb (i.e., 405 nm, curve a), suggesting that Hb in the AZW–SA film has a secondary structure, similar to the native state of Hb, and retains its biological activity.

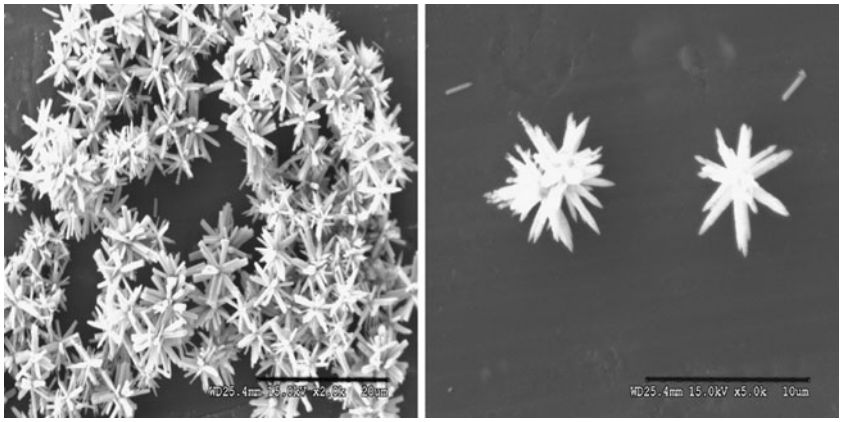

Fig. 1 SEM images of AZW

Direct electron transfer behavior of Hb/AZW–SA/Au

As shown in Fig. 3, AZW–SA/Au (curve a) do not display any peaks under given conditions. However, Hb/AZW–SA/Au (curve b) gives a pair of well-defined redox peaks. This reveals that the acerate ZnO whiskers with large surface area for assembling Hb make more Hb absorbed on electrode surface. Moreover, good biocompatibility of AZW–SA retains the bioactivity of Hb, which is also important in realizing the direct electron transfer between Hb and Au.

From the integration of reduction peak currents and using Faraday's law, the surface concentration of electroactive Hb (Γ) on the Hb/AZW–SA/Au surface could be estimated according to Laviron's equation [22]; the relationship between peak current (I_p) and surface coverage (Γ) is

$$I_P = \frac{n^2F^2vA\Gamma}{4RT} = \frac{nFQv}{4RT}$$

where A is the surface area, n is the number of electrons transferred, Q is the total amount of charge, and F is the Faraday constant. From the CV curves, Q is calculated to be 5.122×10^{-6} C after background correction. Thus, the average surface concentration (Γ) of Hb is calculated to be 4.21×10^{-10} mol/cm^2. The theoretical monolayer coverage for Hb is about 1.89×10^{-11} mol/cm^2 [23]. This suggested that multiple layers of Hb were coated on the electrode. Comparing this calculated value with those reported for Hb surface concentration in other immobilized matrices, such as Ag sol films (2.78×10^{-10} mol/cm^2) [24], meso-Al_2O_3 (1.16×10^{-10} mol/cm^2) [9] is more efficient for Hb immobilization, which could be attributed to the good biocompatibility and large surface area of acerate ZnO whiskers.

Effect of scan rate on direct electron transfer of immobilized Hb

Figure 4 shows the CVs of Hb/AZW–SA/Au in 0.1 mM PBS at different scan rates. With an increasing scan rate

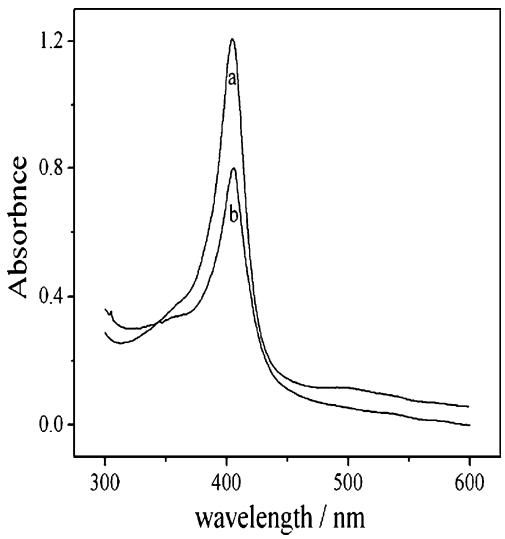

Fig. 2 UV–vis spectra of Hb in solution (**a**) and Hb/AZW (**b**)

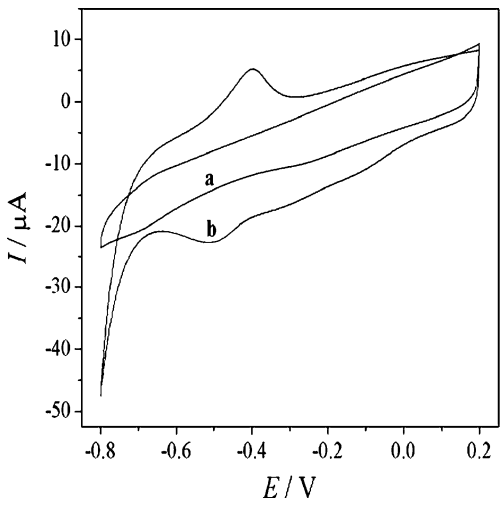

Fig. 3 CVs of bare Au electrode (**a**) and Hb/AZW–SA/Au (**b**) in 0.1 M PBS (pH 7.0), scan rate 100 mV/s

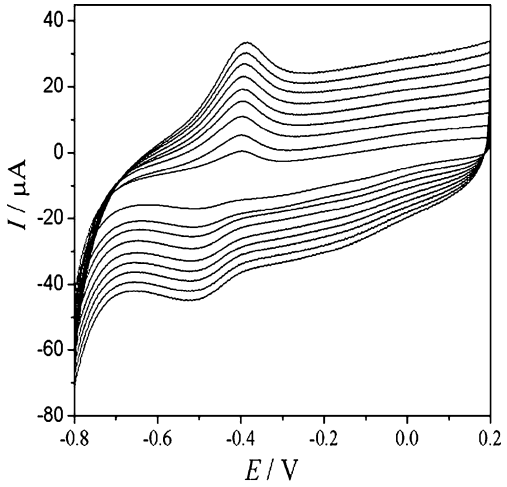

Fig. 4 Cyclic voltammograms of the biosensor at different scan rates (from inner to outer): 50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550 mV/s in 0.1 M PBS (pH 7.0)

ranging from 50 to 450 mV/s, the anodic and cathodic peak potentials of Hb showed a small shift (inset in Fig. 5) and the redox peak currents (Fig. 5) increased linearly, indicating a surface-controlled electrode process.

According to the Laviron equation [23], the transfer coefficient (α) and electron transfer rate constant (k_s) can be estimated by measuring the variation of peak potential with scan rate.

$$\log k_s = \alpha \log(1-\alpha) + \alpha(1-\alpha)\log\alpha - \log\frac{RT}{nF\nu} - \frac{\alpha(1-\alpha)nF\Delta E_P}{2.3RT}$$

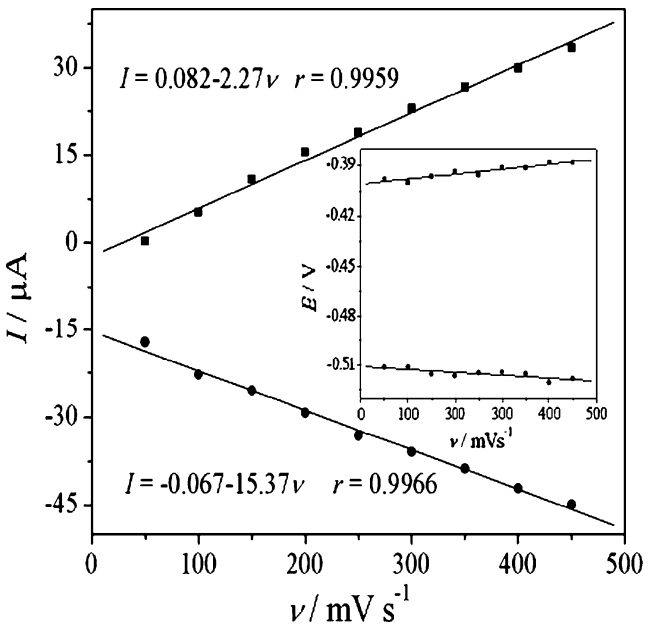

Fig. 5 Plot of peak currents vs. scan rates. *Inset* Various of peak potentials vs. the scan rates

where R is the gas constant, T is the absolute temperature, ΔE_P is the peak potential separation, and ν is the scan rate. The transfer coefficient (α) and electron transfer rate constant (k_s) were calculated to be 0.5 and 2.5 s^{-1}, respectively. The value for electron transfer rate constant is higher than the electron transfer rate constant of Hb immobilized on a CHT/nano-$CaCO_3$ composite film (1.8 s^{-1}) [25] and silica sol–gel film (1.58 s^{-1}) [26]. It exhibited a fast electron transfer process between the immobilized Hb and the underlying electrode due to the presence of the AZW–SA film.

Influence of pH and applied potential on biosensor response

It is well known that pH is a critical parameter of the enzyme activity and the stability in aqueous media [27]. When solution pH changes from 4.5 to 8.5, both the anodic and cathodic peak potentials shift to the negative direction, and the maximum peak currents of Hb occurred at pH 7.0 (Fig. 6). Therefore, the buffer solution of pH 7.0 was selected for further experiments.

The inset in Fig. 6 shows the dependence of the current response to hydrogen peroxide on the applied potential in the range from 0.1 to −0.6 V. Although amperometric current increased gradually when the applied potential shifted from −0.6 to 0.1 V, an applied potential of −0.25 V was selected for the amperometric determination of H_2O_2 because we not only could obtain sufficient current

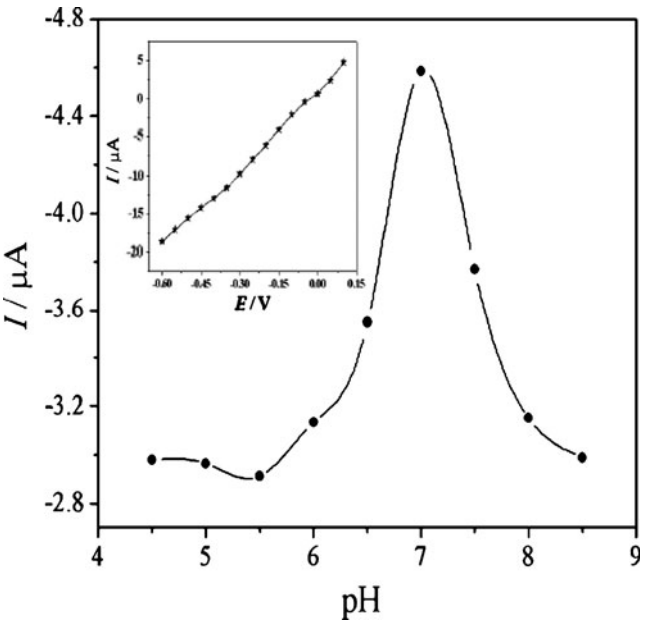

Fig. 6 Cyclic voltammograms of Hb/AZW/Au in 0.1 M PBS at different pH, from *left* to *right* 8.5 to 4.5, at a scan rate of 100 mV/s. *Inset* Amperometric current of the biosensor varies with the applied potential

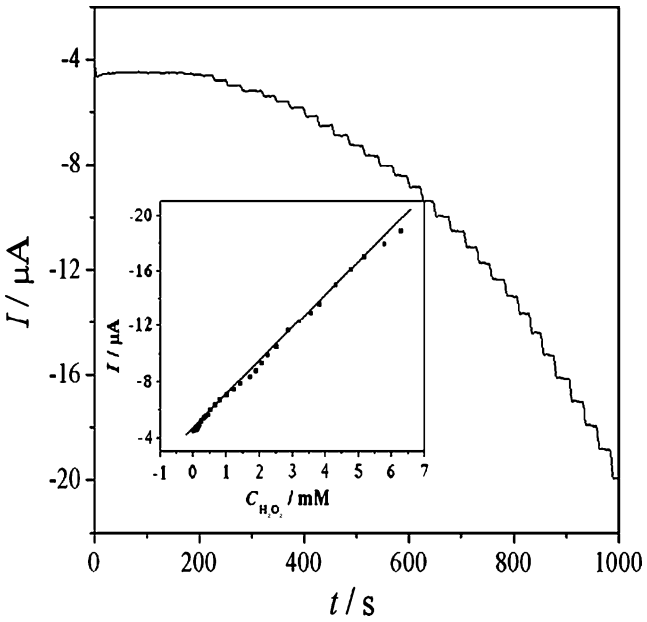

Fig. 7 Typical current–time response of the biosensor on successive injection of different concentration of H_2O_2 into a stirred solution of 0.1 M PBS (pH 7.0) at an applied potential of −0.25 V in the time intervals of 30 s. *Inset* Calibration plot of currents vs. H_2O_2 concentrations

responses but also could minimize the risk for interfering reactions by other electroactive species in the solution at −0.25 V.

Electrocatalytical activity of the Hb/AZW–SA/Au electrode to H_2O_2

Amperometric response of Hb/AZW–SA/Au to the successive additions of H_2O_2 in 0.1 M PBS (pH 7.0) at an applied potential of −0.25 V is illustrated in Fig. 7. It can be observed that as soon as H_2O_2 is added, the background current was changed and the reduction current rises steeply to the reached value. The modified electrode achieves 95% of the steady-state current in <5 s, indicating that the electrocatalytic response is very fast. It shows that the AZW–SA film provides a compatible microenvironment for Hb adsorption. There is a linear relation of the current with a concentration of H_2O_2 between 2.1×10^{-6} and 4.8×10^{-3} M, as shown in the inset of Fig. 7. The linear regression equation is $i(\mu A)=-4.6–2.4\ [H_2O_2](mM)$, with a correlation coefficient of 0.999 (n=33). From the slope of the calibration curve, the detection limit of 7.0×10^{-7} M is estimated at a signal-to-noise ratio of 3.

When the concentration of H_2O_2 is higher than 4.8 mM, a response plateau is observed, showing a typical Michaelis–Menten kinetic mechanism. The Michaelis–Menten constant $\left(K_m^{app}\right)$ gives an indication of the enzyme substrate kinetics. The apparent $\left(K_m^{app}\right)$ constant can be obtained from the Lineweaver–Burk equation [28]:

$$1/I_{ss} = 1/I_{max} + K_M^{app}/I_{max}C$$

where I_{ss} is the steady current, I_{max} is the current measured when H_2O_2 is saturated in PBS solution, and C is the concentration of H_2O_2. The value for this H_2O_2 biosensor was calculated to be 0.8 mM from Lineweaver–Burk equation. This value was smaller than those of Hb in Triton X-100 of 4.27 mM [29], Hb entrapped into nickel oxide film, 1.37 mM [30], and Hb immobilized in mesoporous silicas, 2.87 mM [28], suggesting a higher affinity to H_2O_2 and a higher enzymatic activity to H_2O_2 reduction for the adsorption of Hb into AZW.

Comparison with other hydrogen peroxide biosensor-based different modified electrode materials

The analytical performances of the proposed biosensor were compared with other hydrogen peroxide biosensors reported in the literature. The results were shown in Table 1. In contrast with other H_2O_2 biosensors reported in the last 5 years (Table 1) [31–35], these results proved that the advantages of the biosensor configuration in this work were shown because of the synergic effect of acerate ZnO whiskers and sodium alginate. For example, the linear range, k_s, K_m^{app} and average

Table 1 Analytical properties of H_2O_2 biosensors based on different modified electrode materials

S. No.	Structure of electrode	Γ (mol/cm^2)	k_s (s^{-1})	Linear range (μM)	Detection limit (μM)	K_m^{app}(mM)	References
1	ZnO–MWCNTs/Nafion/GCE	5.82×10^{-11}	1.14	0.2–12	0.084	0.83	[31]
2	ZnO–GNPs–Nafion–HRP/GC			15–1100	9.0	1.76	[32]
3	Mb–ZnO–modified GE	5.2×10^{-10}	1.00	10–180	4.0		[33]
4	HRP-modified screen-printed gold electrode	3.3×10^{-12}	1.0	5–30	2		[34]
5	Ti(III)–TNTs/Hb	1.5×10^{-9}		4.9–1100	1.5	1.02	[35]
6	Hemoglobin/ZnO–chitosan/nano-Au			0.194–1730	0.097	0.075	[36]
7	Hb/AZW–SA/Au	4.21×10^{-10}	2.5	2.1–4800	0.7	0.8	this work

surface concentration (Γ) of biosensor are better than that of most biosensors reported in the references. These indicate that the Hb/AZW–SA/Au electrode is an excellent platform for the detection of H_2O_2. The AZW–SA matrix is facilitated by the suitable biocompatible microenvironment provided by the composite thin film. But the biosensor reported in [36] presents better performance than the present work. It may be caused by the method of modified electrode, ZnO morphology, and electrode materials. However, the process of the Hb/AZW–SA-modified electrode method is simpler than that of [36].

Stability and reproducibility of the Hb/AZW–SA/Au electrode

The stability and reproducibility of the hydrogen peroxide biosensor based on Hb/AZW–SA film were examined. The biosensor could retain the direct electrochemistry of the immobilized Hb at constant current values upon the continuous CV sweep over the potential range from −0.8 to +0.2 V at 0.1 $V s^{-1}$.When stored at 4 °C for over 2 weeks, the biosensor retained 90% of the initial sensitivity to H_2O_2. The good long-term stability can be attributed to the good biocompatibility of the composites, which can provide a favorable microenvironment for Hb to retain its bioactivity. The fabrication reproducibility of ten electrodes, made independently, showed acceptable reproducibility with the relative standard deviations of 3.4% for the current determinations of 3.5 μM H_2O_2. These indicate an efficient and reproducible immobilization process of Hb in the AZW–SA film.

Conclusion

In this paper, hemoglobin was successfully immobilized at Au electrode modified with electrodeposited acerate ZnO whiskers and sodium alginate. When Hb is immobilized on the Au surface by AZW–SA, a quasi-reversible CV response for Hb Fe(III)/Fe(II) is obtained. The suggested electrodeposited method provides a mild immobilization process for Hb and a biocompatible microenvironment around the Hb, thus can retain its biological activity as a mimetic enzyme. Immobilized Hb in the film displayed good electrocatalytic response to H_2O_2. Good reproducibility and stability of this modified electrode provided a novel and promising biosensor based on Hb co-immobilized with nanoparticles for determining H_2O_2.

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