

## Direct electron transfer of hemoglobin in layered $\alpha$ -zirconium phosphate with a high thermal stability

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### Abstract

A heme protein hemoglobin (Hb) was reacted with preexfoliated layered  $\alpha$ -zirconium phosphate ( $\alpha$ -ZrP) platelets. An X-ray diffraction (XRD) pattern of small range showed that the exfoliated  $\alpha$ -ZrP platelets reassembled after the addition of Hb molecules, with the protein intercalated between the layers. UV–Vis and Fourier transform infrared (FTIR) spectra analysis displayed that no significant denaturation occurred to the intercalated protein. The bioactivity of Hb was also investigated by testing the electrochemical properties of the Hb/ $\alpha$ -ZrP composite. Results showed that the intercalation of Hb into the layered material not only improved the thermal stability of Hb but also enhanced the direct electron transfer ability between protein molecules and electrode. The protein still showed bioactivity after treatment at a temperature as high as 85 °C. A pair of well-defined redox peaks at approximately  $-0.37$  and  $-0.32$  V was observed on the cyclic voltammograms (CVs) of the Hb/ $\alpha$ -ZrP composite modified electrode, and the electrode reactions showed a surface-controlled process with a single proton transfer. The resultant biosensor constructed by the Hb/ $\alpha$ -ZrP composite displayed an excellent response to the reduction of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) with good reproducibility.

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**Keywords:** Hemoglobin; Direct electron transfer; Layered  $\alpha$ -zirconium phosphate; Intercalation; Hydrogen peroxide

The direct electron transfer between biomacromolecules and electrode surface has been a focus during recent years. The research on the mechanism of direct electron transfer from protein to electrode surface and the electrocatalytic reactions can serve as a model to understand the electron transfer behavior in biological systems that may elucidate the relationship between their structures and biological functions [1]. Among the biomacromolecules, the electrochemical biosensors constructed on the basis of the direct electron transfer between redox proteins and the electrode have been developed increasingly in the fields of medicine, biotechnology, environmental monitoring, and so on [2–5].

Heme proteins are important redox proteins that contain the porphyrin complex of iron(II) or heme(III) as a

prosthetic group. The direct electrochemistry of these proteins can establish a desirable model for fundamental study of the redox behavior of the proteins in biological systems. Hemoglobin (Hb)<sup>1</sup> is a soft globular heme protein. It has four electroactive iron hemes, a molar mass of approximately  $64,500 \text{ gmol}^{-1}$ , and protein dimensions of  $5.3 \times 5.4 \times 6.5 \text{ nm}$  [6]. The isoelectric point (pI) of Hb is approximately 6.8 to 7.0 [7]. In general, Hb is an ideal model molecule for the study of electron transfer reactions of heme proteins and also for biosensing and electrocatalysis [8]. However, it usually is difficult to directly transfer

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<sup>1</sup> Abbreviations used: Hb, hemoglobin;  $\alpha$ -ZrP,  $\alpha$ -zirconium phosphate;  $\text{H}_2\text{O}_2$ , hydrogen peroxide; TBAOH, tetrabutylammonium hydroxide; PBS, phosphate-buffered solution; e- $\alpha$ -ZrP, exfoliated  $\alpha$ -ZrP; GCE, glassy carbon electrode; TEM, transmission electron micrograph; SEM, scanning electron microscope; FTIR, Fourier transform infrared; XRD, X-ray diffraction; CV, cyclic voltammogram; RSD, relative standard deviation.

an electron between Hb and the conventional electrode, and great efforts have been made on this aspect [9–11].

For proteins, immobilization can make them retain their activities better than their free state. This is because immobilization on solid supports permits highly selective catalysis to be performed for enzymes by using materials that are chemically and mechanically robust and readily separated from reaction mixtures [12,13]. It is also favorable for the enhancement of stability of enzyme on electrode.

During recent years, layered materials have attracted great attention for their application in the immobilization of proteins. The “flexible pores” and interlayer galleries in layered materials can be used to hold the dimension of guests, making them quite suitable to immobilize proteins with different dimensions [14]. Different kinds of layered materials have been reported as supporting matrices for proteins [15–18].

Among those materials, layered zirconium phosphate has been studied extensively, especially  $\alpha$ -zirconium phosphate ( $\alpha$ -Zr(HPO<sub>4</sub>)<sub>2</sub>•*n*H<sub>2</sub>O, abbreviated as  $\alpha$ -ZrP).  $\alpha$ -ZrP is a well-characterized layered material with hydrophilic hydroxyl groups on its two-dimensional lamellar surface (1 OH/24 Å<sup>2</sup>) [18,19]. The material is thermally stable and chemically inert in neutral or acidic media. The layers of  $\alpha$ -ZrP can be readily expanded to accommodate both small and large guest molecules, and this is a bright advantage for the immobilization of proteins over other materials.  $\alpha$ -ZrP has a large surface area on exfoliation of the lamellae [20], and it can provide anionic surfaces (up to one negative charge per phosphate) for binding. The hydrophilic nature of  $\alpha$ -ZrP may play an important role in stabilizing the surface-bonded protein, and the narrow galleries can protect the bound proteins from microbial degradation. It has also been reported that the OH groups of  $\alpha$ -ZrP can be replaced by several functional groups such as phenyl, 2-carboxyethyl, and sulfophenyl [20], providing different surface characteristics for the interaction with proteins. Until now, some proteins have been successfully immobilized into the galleries of  $\alpha$ -ZrP [16,21].

Because  $\alpha$ -ZrP can provide a friendly microenvironment to retain proteins' bioactivity and prevent them from denaturation, it is used in this study to realize the direct electron transfer between Hb and the electrode. An attempt to apply  $\alpha$ -ZrP immobilized with Hb in direct electrochemistry has been made [22], but the direct electron transfer of the bound Hb in  $\alpha$ -ZrP was hard to realize. The reason may be that Hb was immobilized onto the outer surface of  $\alpha$ -ZrP and this provided only weak stability for the protein. Instead of the method used in the literature [22], a pre-exfoliation method reported by Mallouk and coworkers was used here to immobilize the protein into the galleries of the layered material to enhance the stability of protein [23]. The same method has also been used for protein binding by others [16,21].

The accurate determination of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) plays an important role in many fields such as

industry, clinical control, and environmental protection [23,24]. Compared with the traditional techniques such as titrimetry and spectrophotometry [25,26], the electrochemical method is fast and easy and has obvious advantages for the determination of H<sub>2</sub>O<sub>2</sub> [27,28]. Heme proteins have peroxidase activity and can be used to reduce H<sub>2</sub>O<sub>2</sub> through electrochemical catalysis. In this work, a sensor for the reduction of H<sub>2</sub>O<sub>2</sub> was constructed by modifying the electrode with the resultant Hb/ $\alpha$ -ZrP composite.

## Materials and methods

### Reagents

Bovine heart Hb was purchased from Sigma and used without further purification. Tetrabutylammonium hydroxide (TBAOH) aqueous solution (10 wt%) was purchased from Shanghai Chemical Reagent Company. H<sub>2</sub>O<sub>2</sub> (30 wt% aqueous solution) was obtained from Shanghai Biochemical Reagent Company. All other reagents were of analytical grade and used as received.

### Synthesis and exfoliation of $\alpha$ -ZrP

$\alpha$ -ZrP was synthesized according to the literature with minor modifications [18,19]. In a typical process, ZrOCl<sub>2</sub> (10 g, 31 mmol) was added to phosphoric acid (17 g, 172 mmol, 9 M) and the mixture was heated at 90 °C for 24 h. The resulting white product was washed with water and acetone and then dried overnight at 60 °C.

Exfoliation of  $\alpha$ -ZrP was performed as follows. After 0.1 g of  $\alpha$ -ZrP was added to 5 ml of distilled water and a stoichiometric amount of TBAOH, the resulting suspension was sonicated for approximately 1 h and used without further treatment.

### Intercalation of Hb and fabrication of biosensor

For the intercalation of Hb, 2 ml of the stock solution of Hb (2 mg/ml, 0.1 M phosphate-buffered solution [PBS], pH 7.0) was mixed with 2 ml of the exfoliated  $\alpha$ -ZrP (e-  $\alpha$ -ZrP, ~1.4% by weight) and then the mixture was equilibrated for 24 h at room temperature and centrifuged to collect the solid. The resulting Hb/ $\alpha$ -ZrP composite was used for further testing.

Glassy carbon electrode (GCE) was first polished with 1.0, 0.3, and 0.05  $\mu$ m alumina powder successively, followed by rinsing thoroughly with doubly distilled water. The polished electrode was then sonicated in acetone and doubly distilled water and finally was allowed to dry at room temperature. The Hb/ $\alpha$ -ZrP composite mentioned above was resuspended in 1 ml of water, and 5  $\mu$ l of this suspension was deposited onto the electrode surface. The electrode was then left to dry at 4 °C for at least 24 h. The sensor was stored under the same conditions when not in use.

### Apparatus and measurements

Powder X-ray diffraction patterns were obtained on an ARL X'TRA X-ray diffractometer using  $\text{CuK}\alpha$  radiation. Transmission electron micrograph (TEM) patterns were recorded on a JEOL JEM 200CX transmission electron microscope. Scanning electron microscope (SEM, LEO1530VP) images were taken on a LEO-1530VP field emission scanning electron microscope. UV–Vis absorption spectra were recorded on a Shimadzu UV-2401PC spectrophotometer. Fourier transform infrared (FTIR) spectroscopy measurements were made on a Bruker Fourier transform spectrometer model VECTOR22 using KBr pressed discs. Cyclic voltammetric and amperometric experiments were conducted with a CHI660B workstation (Shanghai Chenhua). All experiments were carried out using a conventional three-electrode system, where GCE modified with Hb/ $\alpha$ -ZrP was used as working electrode, a platinum wire was used as auxiliary electrode, and a saturated calomel electrode was used as reference electrode. All solutions were deoxygenated by highly pure nitrogen before and during the measurements.

### Results and discussion

In this part, the interaction between Hb and e- $\alpha$ -ZrP was investigated by X-ray diffraction (XRD), SEM, UV–Vis, and FTIR spectra analysis. The bioactivity of Hb intercalated into the layers of  $\alpha$ -ZrP was then studied by testing various electrochemical properties of the Hb/ $\alpha$ -ZrP composite modified electrode.

#### XRD and SEM analysis

The powder XRD pattern of the as-prepared  $\alpha$ -ZrP matched well with that reported previously [29].

The layers of  $\alpha$ -ZrP can be exfoliated to form individual platelets and lose the original layered structure, and then the guests can be adsorbed onto the surface of these platelets. These platelets can be reassembled under appropriate conditions to form stacks of  $\alpha$ -ZrP platelets, trapping the intercalated guests in the galleries at the same time. The XRD pattern of small range shown in Fig. 1 displays a strong peak. Because there is no peak in the original and e- $\alpha$ -ZrP, the peak in Fig. 1A is due to the reassembly of the  $\alpha$ -ZrP platelets after the addition of Hb. The interlayer space can be calculated to be 6.8 nm according to this peak, which is greater than the protein dimensions of Hb ( $5.3 \times 5.4 \times 6.5$  nm), suggesting that Hb is successfully intercalated into the galleries of  $\alpha$ -ZrP. In other words, the  $\alpha$ -ZrP platelets are reassembled on the Hb molecules, so the layered structure remained in such a way with change only in interlayer distance.

Morphology of the Hb/ $\alpha$ -ZrP film on the electrode was investigated by SEM. As shown in Fig. 2A, the surface of the Hb/ $\alpha$ -ZrP film is uniform at a low magnification, implying that the e- $\alpha$ -ZrP comes into being homogeneously

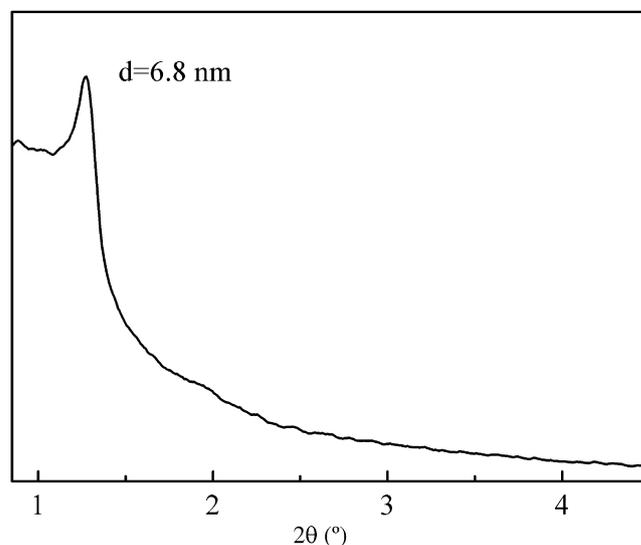


Fig. 1. XRD pattern of Hb/ $\alpha$ -ZrP composite.

layers with Hb molecules intercalated. When shown at a high magnification, a typical layered structure can be observed (Fig. 2B). All of this indicates that the e- $\alpha$ -ZrP is reassembled after reacting with Hb, in accordance with the results of XRD.

#### UV–Vis and FTIR spectra analysis

UV–Vis spectroscopy is a useful tool for monitoring the possible change of the Soret absorption band in the heme group region [30]. The band shift may provide some information for possible denaturation in heme protein, particularly that of conformational change. Fig. 3A shows the UV–Vis spectra of preexfoliated  $\alpha$ -ZrP, Hb/ $\alpha$ -ZrP, and Hb in 0.1 M PBS (pH 7.0). Free Hb and Hb/ $\alpha$ -ZrP have Soret absorptions at 408 and 412 nm, indicating that no significant denaturation occurred to protein after intercalation into  $\alpha$ -ZrP.

The interaction between  $\alpha$ -ZrP and Hb could be demonstrated with the FTIR spectra of  $\alpha$ -ZrP and Hb/ $\alpha$ -ZrP. It can be seen from the spectrum of  $\alpha$ -ZrP (Fig. 3B) that the band at  $1620\text{ cm}^{-1}$  is attributed to the surface-adsorbed water and hydroxyl groups [31]. The bands around  $1650$  and  $1550\text{ cm}^{-1}$  correspond to the amide I and amide II infrared adsorption bands of Hb and can provide detailed information on secondary structure of the polypeptide chain [32]. The amide I band ( $1700\text{--}1600\text{ cm}^{-1}$ ) is attributed to C=O stretching vibration of peptide linkages in the backbone of protein. The amide II band ( $1620\text{--}1500\text{ cm}^{-1}$ ) results from a combination of N–H bending and C–N stretching. On the intercalation of Hb into  $\alpha$ -ZrP, the bands corresponding to the amide I and amide II groups were retained, suggesting that the native structure is retained.

The results of XRD, SEM, and FTIR spectra have also been confirmed by other reports [16,17], indicating that

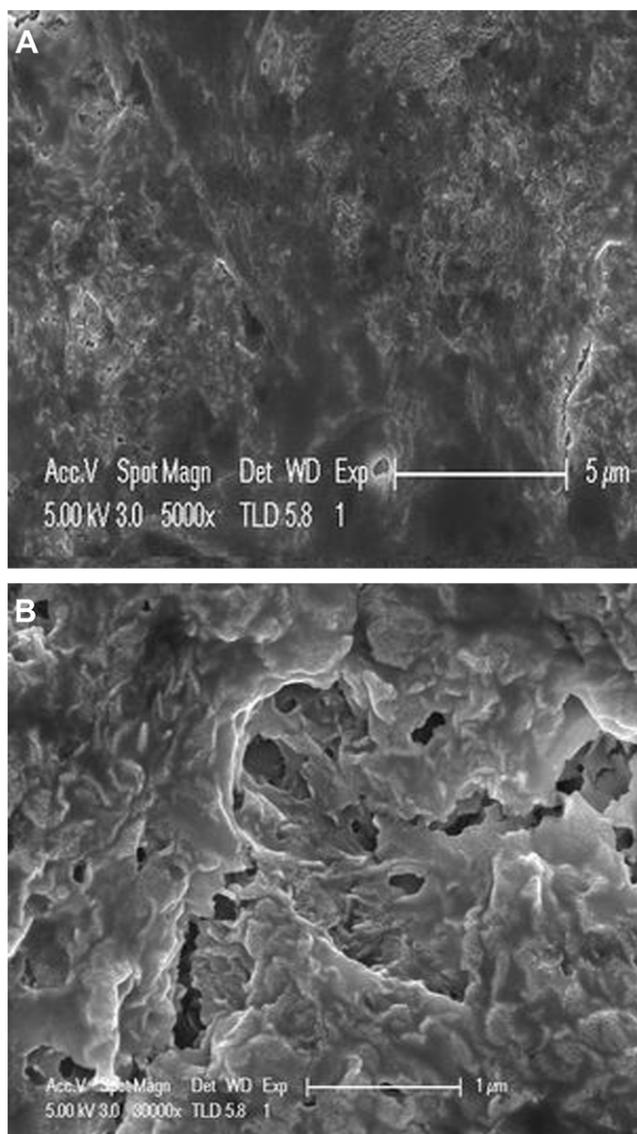


Fig. 2. SEM image of Hb/ $\alpha$ -ZrP film at low magnification of 5000 $\times$  (A) and high magnification of 30,000 $\times$  (B).

such a method of exfoliation can be widely used for the intercalation of proteins and biomolecules.

#### Direct electrochemistry of Hb/ $\alpha$ -ZrP modified electrode

As mentioned in the introductory paragraphs, intercalating biomolecules into the galleries of layered materials have been investigated extensively. However, the electrochemical properties of the resultant biomolecules/layered material composite have been reported rarely. In this study, electrochemical methods were used to test the bioactivity of Hb intercalated into  $\alpha$ -ZrP, and this shows a new aspect for the application of layered materials.

The cyclic voltammograms (CVs) of different electrodes at 100 mV/s are given in Fig. 4. No peak appeared at the electrode modified by preexfoliated  $\alpha$ -ZrP, indicating that  $\alpha$ -ZrP is nonelectroactive. When the electrode is modified

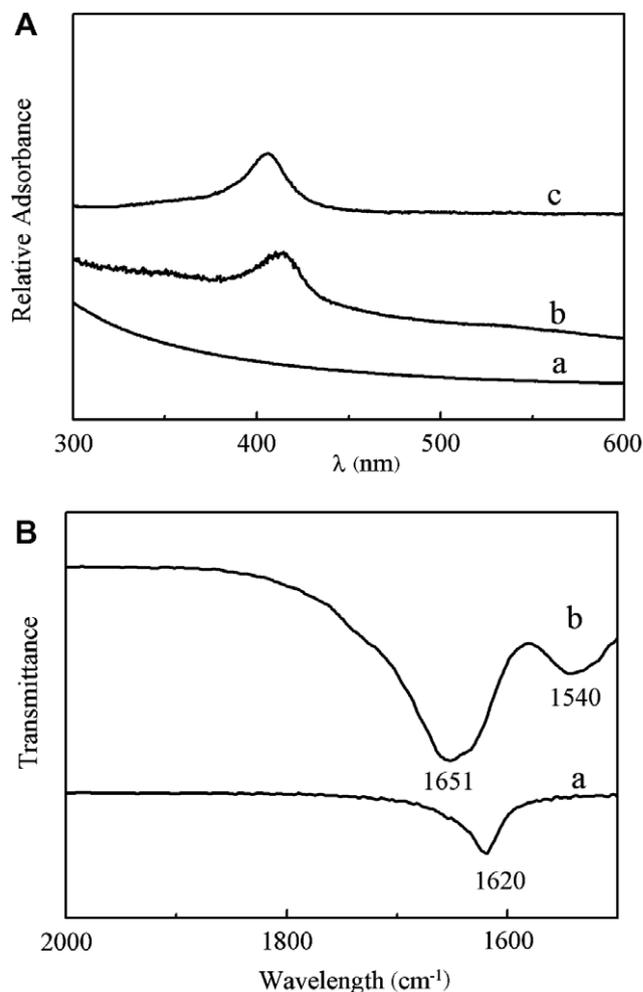


Fig. 3. (A) UV-Vis spectra of preexfoliated  $\alpha$ -ZrP (a), Hb/ $\alpha$ -ZrP (b), and free Hb (c) in 0.1 M PBS (pH 7.0). (B) FTIR spectra of  $\alpha$ -ZrP (a) and Hb/ $\alpha$ -ZrP (b).

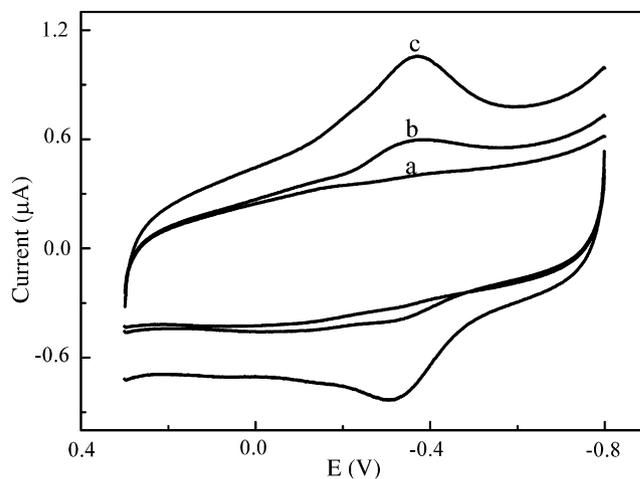


Fig. 4. Cyclic voltammograms of preexfoliated  $\alpha$ -ZrP/GCE (a), Hb/GCE (b), and Hb/ $\alpha$ -ZrP/GCE (c) in 0.1 M PBS (pH 7.0) at 100 mV/s.

with Hb, only an irreversible and small reduction peak is observed and the reduction current decreased with the cycle numbers, suggesting that the direct electron transfer

is difficult between Hb and electrode. However, at the Hb/ $\alpha$ -ZrP modified electrode, a couple of well-defined redox peaks are observed at  $-0.37$  and  $-0.32$  V. These peaks are located close to the characteristic potential of the heme Fe(III)/Fe(II) couples of the proteins in the literature [33]. The results present strong evidence that the direct electron transfer between Hb and GCE can be achieved after intercalation of Hb into the layered  $\alpha$ -ZrP and that the immobilization may have more favorable orientation and facilitate the direct electron transfer between Hb and electrode. This may be due to the particular electron transferring channels that  $\alpha$ -ZrP provides.

Typical CVs of Hb/ $\alpha$ -ZrP at different scan rates are shown in Fig. 5. The electrode modified with the Hb/ $\alpha$ -ZrP composite shows well-defined peaks at different scan rates from 20 to 450 mV. With the increase of scan rates, the redox peak currents of the Hb increased linearly and the peak-to-peak separation also increased (inset in Fig. 5B), indicating a surface-controlled process.

For thin-layer electrochemistry, integration of CV peak can give the total amount of charge ( $Q$ ) passed through the electrode for reduction or oxidation of electroactive species in the thin film. Its surface concentration ( $\Gamma^*$ ) can be calculated from the following equation:

$$\Gamma^* = Q/nFA,$$

where  $n$  is the number of electrons transferred,  $F$  is Faraday's constant, and  $A$  is the electrode area. According to the equation, the average surface coverage of Hb was calculated to be  $1.3 \times 10^{-10}$  mol/cm<sup>2</sup> for the Hb/ $\alpha$ -ZrP modified GCE. The value is larger than the theoretical monolayer coverage of Hb ( $\sim 1.89 \times 10^{-11}$  mol/cm<sup>2</sup>) on the basis of the crystallographic dimensional structure of Hb and assuming that the biomolecule adopts an orientation with the long axis parallel to the electrode surface [34]. The bigger surface coverage can be ascribed to the ex-

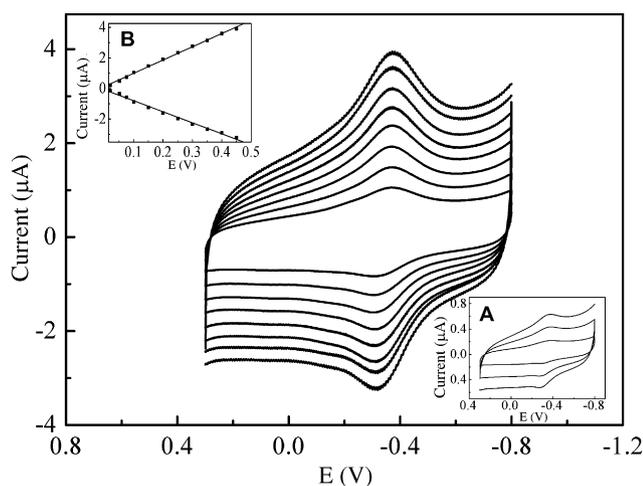


Fig. 5. Cyclic voltammograms of Hb/ $\alpha$ -ZrP modified electrode in 0.1 M PBS (pH 7.0) at 20, 50, 80, 100, 150, 200, 250, 300, and 350 mV/s. Insets: Cyclic voltammograms of this system at 20, 50, and 80 mV/s (A) and plot of peak current versus scan rate (B).

panded interspace to hold more Hb molecules, indicating that multilayers of intercalated Hb in the layered film participate in the electron transfer process.

Small peak-to-peak separation always indicates a fast electron transfer rate. The electron transfer rate constant  $k_s$  can be estimated by the Laviron equation [35]:

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

where  $\alpha$  is the charge transfer coefficient,  $R$  is the gas constant,  $T$  is the absolute temperature,  $\Delta E_p$  is the peak potential separation, and  $v$  is the scan rate. A graph of the peak potential versus the logarithm of the scan rate yields a straight line, from the slope of which a charge transfer coefficient of 0.45 was estimated for Hb. The peak-to-peak separations were 66, 74, 95, and 110 mV at scan rates of 100, 150, 200, and 250 mV/s, respectively, giving an average  $k_s$  value of  $1.85 \pm 0.51$  s<sup>-1</sup>. The value is much larger than the values reported previously [10,36,37]. This can also be explained by the particular galleries of  $\alpha$ -ZrP that accelerate the electron transfer between Hb and the electrode, indicating the excellent biocompatibility of  $\alpha$ -ZrP for biomolecules and its application in electrochemistry.

#### Influence of solution pH on the direct electron transfer of Hb

A pair of stable and well-defined redox peaks was obtained for intercalated Hb in different pH solutions (Fig. 6). An increase of solution pH caused a negative shift in both cathodic and anodic peak potentials. All changes in voltammetric peak potentials and currents with pH were reversible. For example, the CV for the Hb/ $\alpha$ -ZrP at pH 9.0 was reproduced after immersion in pH 4.0 buffer and then returned to the pH 9.0 buffer. The increase of pH also resulted in a shift of the formal potential ( $E^0$ ) toward

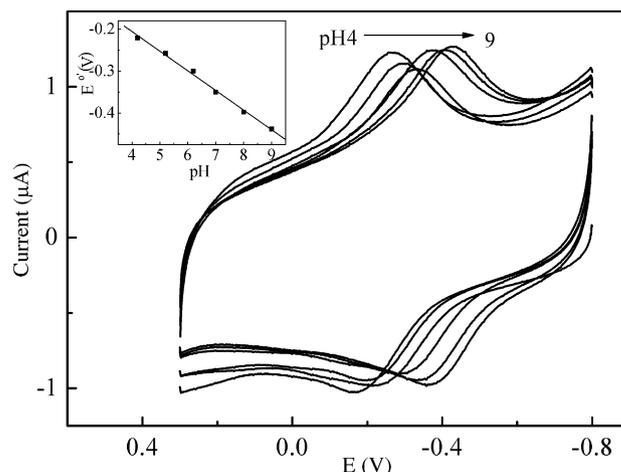
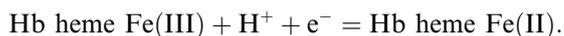


Fig. 6. Cyclic voltammograms of Hb/ $\alpha$ -ZrP/GCE in 0.1 M PBS with different pH values at 100 mV/s. Inset: Plot of formal potentials versus pH.

negative accordingly, in agreement with that reported previously [37]. The  $E^0$  of the heme Fe(III)/Fe(II) redox couple for the Hb/ $\alpha$ -ZrP modified electrodes showed a linear relationship, with pH in the range of 4.0 to 9.0 and a slope of  $-46.8 \text{ mV pH}^{-1}$ . This value is a bit smaller than  $-57.8 \text{ mV pH}^{-1}$  at  $18^\circ\text{C}$  for a reversible one-proton coupled single-electron transfer during electrochemical reduction [38]. This may be due to the effect of the protonation states of trans ligands to the heme iron and amino acids around the heme or to the protonation of water molecules coordinated to the center [39] that may exist in different states under different pH values. Thus, the reaction equation for the electrochemical reduction of Hb may be described as follows [40]:



#### Influence of temperature on thermal stability of Hb

The activity of protein depends greatly on the temperature. Fig. 7 displays the effect of temperature on the activity of Hb after equilibrating the samples for 5 min at the desired temperature in a thermal bath. Due to the denaturation of Hb, no peak can be observed at the Hb/graphite electrode above  $50^\circ\text{C}$ . Usually, Hb will denature at approximately  $73^\circ\text{C}$  in solution [41]. However, the peak currents of the Hb/ $\alpha$ -ZrP electrode increased from room temperature to  $85^\circ\text{C}$  and reached a maximum at  $85^\circ\text{C}$  in our research. When the temperature was above  $85^\circ\text{C}$ , the peak currents began to decrease, indicating the denaturation of the protein. The temperature is a bit lower than that in other investigations [41], but much higher than the denaturation of the protein reported in the literature [42–44]. The high thermal stability may be explained by the structure of  $\alpha$ -ZrP, which confines the space that the Hb molecules occupy and, hence, prevents the denaturation of the protein.

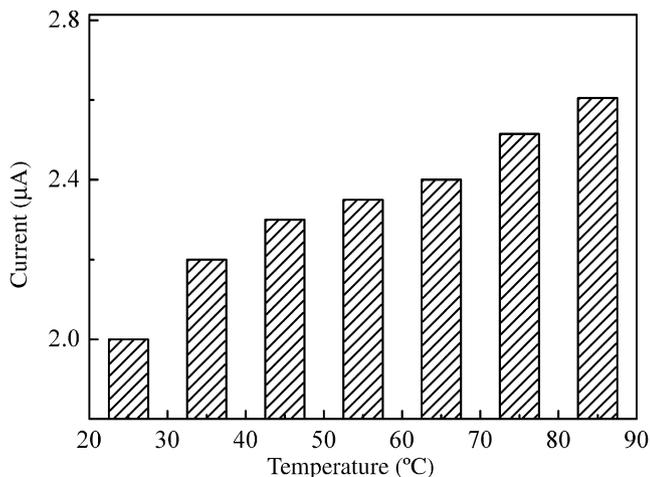


Fig. 7. Effect of temperature on the reduction peak current in 0.1 M PBS (pH 7.0) at 100 mV/s (from left to right: 25, 35, 45, 55, 65, 75, and  $85^\circ\text{C}$ ).

#### Electrocatalysis of Hb/ $\alpha$ -ZrP modified electrode to the reduction of $\text{H}_2\text{O}_2$

Heme proteins have peroxidase activity and can be used to reduce  $\text{H}_2\text{O}_2$  through electrochemical catalysis. Based on the excellent electrochemical behaviors of the Hb/ $\alpha$ -ZrP composite, it was immobilized on the surface of GCE and applied to construct a sensor.

The CVs of Hb/ $\alpha$ -ZrP and  $\alpha$ -ZrP modified electrode in 0.1 M PBS (pH 7.0) before and after the addition of  $\text{H}_2\text{O}_2$  are shown in Fig. 8. No current is observed on  $\alpha$ -ZrP modified electrode. However, the reduction peak current increased and the anodic peak current decreased dramatically with the addition of  $\text{H}_2\text{O}_2$  on the Hb/ $\alpha$ -ZrP modified electrode. Moreover, the currents of the reduction peaks increased with the increase of  $\text{H}_2\text{O}_2$  concentration, indicating a typical electrocatalytic reduction process. The reduction peak currents have a linear response to  $\text{H}_2\text{O}_2$  concentration in the range of 0.2 to  $10.8 \mu\text{M}$  with a detection limit of  $0.07 \mu\text{M}$  ( $n = 12$ ,  $R = 0.994$ ) (inset in Fig. 8). The relative standard deviation (RSD) of the peak current in six successive determinations at an  $\text{H}_2\text{O}_2$  concentration of  $1 \mu\text{M}$  was 3.62% for Hb/ $\alpha$ -ZrP modified GCE.

When the concentration of  $\text{H}_2\text{O}_2$  was greater than  $10.8 \mu\text{M}$ , a response plateau was observed, showing a typical Michaelis–Menten kinetic mechanism. The apparent Michaelis–Menten constant ( $K_m^{\text{app}}$ ) was calculated to evaluate the catalytic activity of intercalated protein by Lineweaver–Burk equation [45]:

$$1/I_{\text{ss}} = 1/I_{\text{max}} + K_m^{\text{app}}/I_{\text{max}}C,$$

where  $I_{\text{ss}}$  is the steady current after the addition of substrate (which can be obtained from amperometric experiments),  $C$  is the bulk concentration of the substrate, and  $I_{\text{max}}$  is the maximum current measured under the saturated

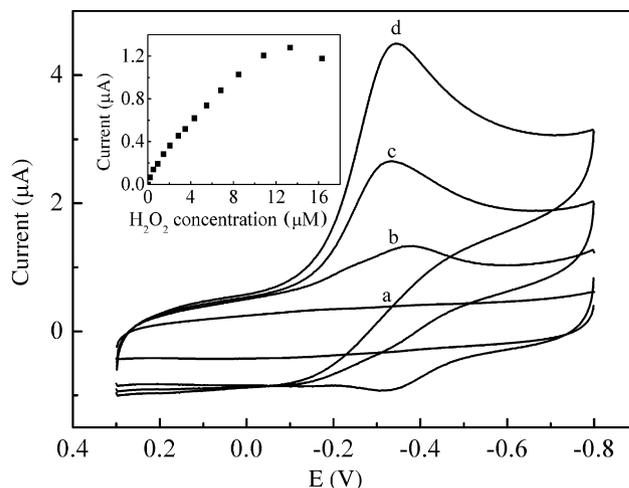


Fig. 8. Cyclic voltammograms of preexfoliated  $\alpha$ -ZrP/GCE with  $5 \times 10^{-4} \text{ M}$   $\text{H}_2\text{O}_2$  (a) and Hb/ $\alpha$ -ZrP/GCE without  $\text{H}_2\text{O}_2$  (b) and with different  $\text{H}_2\text{O}_2$  concentrations at 100 mV/s in 0.1 M PBS (pH 7.0):  $1 \times 10^{-4} \text{ M}$  (c) and  $2 \times 10^{-4} \text{ M}$  (d). Inset: Plot of catalytic current versus  $\text{H}_2\text{O}_2$  concentration.

substrate condition. The apparent Michaelis–Menten constant ( $K_m^{app}$ ), which gives an indication of the enzyme–substrate kinetics, was determined to be 123  $\mu$ M for the Hb/ $\alpha$ -ZrP composite modified electrode (calculated from the linear range in the inset in Fig. 8). The value is much smaller than those reported previously [15,45], suggesting a higher affinity to  $H_2O_2$  and a higher enzymatic activity to  $H_2O_2$  reduction for the intercalation of Hb into  $\alpha$ -ZrP.

### Stability and reproducibility

Additional experiments were carried out to test the reproducibility and stability. No obvious change was found after the Hb/ $\alpha$ -ZrP modified electrode was immersed in PBS and stored in a refrigerator at 4 °C for 20 h. The Hb electrode could keep 95% of its initial response to  $H_2O_2$  within 2 weeks. Thus, the intercalation into  $\alpha$ -ZrP is very efficient to retain the bioactivity of Hb. Because  $\alpha$ -ZrP is chemically inert in neutral or acidic media, the electrode modified by the Hb/ $\alpha$ -ZrP composite may be chemically stable in such environments. However, the mechanical stability might not be high due to the intercalation of Hb inside the galleries.

### Conclusion

The direct electron transfer between Hb and electrode was realized by intercalating the protein into the preexfoliated  $\alpha$ -ZrP platelets. The intercalated Hb still has bioactivity even after treatment at a high temperature up to 85 °C. The reason may be that the preexfoliated  $\alpha$ -ZrP platelets can be reassembled under appropriate conditions to form stacks of  $\alpha$ -ZrP with the addition of Hb, and the interaction between protein and matrix is weak under the conditions studied here. This is also favorable for the protein to retain its bioactivity. The fast electron transfer rate and excellent catalytic ability to the reduction of  $H_2O_2$  also show that the protein retains its bioactivity well. The intercalation of proteins into  $\alpha$ -ZrP provides a new platform for the realization of direct electron transfer and the construction of biosensors.

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