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Studies on direct electron transfer and biocatalytic properties of hemoglobin in polyacrylonitrile matrix

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Abstract

The direct electrochemistry of hemoglobin (Hb) immobilized in polyacrylonitrile (PAN) modified glassy carbon electrode was described. The protein-PAN film exhibited a pair of well-defined and quasi-reversible cyclic voltammetric peaks for Hb Fe(III)/Fe(II) redox couple in a pH 7.0 phosphate buffer. The formal potential of Hb heme Fe(III)/Fe(II) couple varied linearly with the increase of pH in the range of 5.0–9.0 with a slope of 54 mV pH^{-1}, which implied that a proton transfer was accompanied with each electron transfer in the electrochemical reaction. Position of Soret absorption band of Hb-PAN film suggested that the Hb kept its secondary structure similar to its native state in the PAN matrix. The Hb in PAN matrix acted as a biologic catalyst to catalyze the reduction of hydrogen peroxide. The electrocatalytic response showed a linear dependence on the H_2O_2 concentration ranging from 8.3×10^{-6} to 5×10^{-4} mol L^{-1} with a detection limit of 8.3×10^{-6} mol L^{-1} at 3 σ. The apparent Michaelis–Menten constant K_M^{app} for H_2O_2 sensor was estimated to be 0.9 mmol L^{-1}.

Keywords: Direct electrochemistry; Hemoglobin; Polyacrylonitrile; Electrocatalysis

1. Introduction

Since the pioneered work of Hill and Eddowes in the last 1970s [1], the direct electron transfer of redox biomolecules at solid matrices and related biosensing (the so-called "third-generation" biosensors [2]) have received more and more significant attention. The investigations on direct electron transfer process of proteins and underlying electrodes can not only cast the light on the mechanisms of redox transformations between redox biomolecules in biocatalysis and metabolic process involving electron transport in biological systems, but also provide us a platform for fabricating biosensors, enzymatic bioreactors, and biomedical devices [3].

Among various heme proteins, hemoglobin is a desirable model molecule for the study of electron transfer reactions of heme enzymes because of its commercial availability, moderate cost and a known and documented structure. Unlike some other small heme proteins such as cytochromes, it, however, is difficult for Hb to exhibit heterogeneous electron transfer process in most cases, which means that the electron transfer of Hb is very slow [4]. This is ascribed to its extended three-dimensional structure and resulting inaccessibility of the electroactive centers as well as its strong adsorption onto the electrode surface for subsequent passivation. Facilitation of the electron transfer between Hb and electrode is very challenging. Thus, numerous efforts have been devoted to improve the electron transfer characteristics by using insoluble surfactants [5], biopolymers [6], hydrogel polymers [7,8] and nanoparticles [9–11].

Polyacrylonitrile synthesized by single rare-earth catalyst030$Y(OAr)_3$ can be easily fabricated into microporous film with the pore size, which was proved to be suitable for enzyme immobilization [12,13]. In our previous work, we have successfully utilized this polymer to construct glucose biosensor [12] and phenol sensor [13], respectively. In this present paper, the simple cast method was used to immobilize Hb into PAN films on glassy carbon electrode (GCE). To our best knowledge, this is the first report of direct electrochemistry of heme proteins incorporated in this electrochemically inert PAN film. The protein film showed a quasi-reversible cyclic voltammetric peak pair for the heme Fe (III)/Fe(II) redox couple. The Hb-PAN film was characterized by

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electrochemical and spectroscopic techniques. Hydrogen peroxide can be electrochemically catalyzed by the protein-PAN film.

2. Experimental

2.1. Reagents

Bovine erythrocyte hemoglobin (Hb) was obtained from Fluka. It was used as received. Polyacrylonitrile (PAN) (viscosity average molecular weight M_η is 28 000) was synthesized by the polymerization of acrylonitrile with single rare-earth catalyst, $Y(OAr)_3$ [14]. All other chemicals were of analytical grade and used without further purification. H_2O_2 was freshly prepared before being used. Buffers were 0.1 mol L^{-1} Na_2HPO_4 and NaH_2PO_4 and its pH was adjusted with H_3PO_4 or NaOH solutions, Twice-distilled water was used throughout the experiment.

2.2. Construction of PAN film immobilized Hb electrode

The PAN suspension (2 mg mL^{-1}) was prepared by dispersing PAN into deionized water by ultrasonication. Hb was dissolved in 0.01 mol L^{-1} PBS with a concentration of 5 mg mL^{-1}. Prior to use, a glassy carbon electrode (GCE) was polished on a polishing cloth with alumina of successively smaller particles (1.0 μm diameter). Then the electrode was cleaned by ultrasonication in deionized water. To obtain good cyclic voltammetric responses of Hb-PAN/GCE, the concentration and the mass ratio of Hb and PAN were optimized in control experiments. Typically, 10 μl of mixture containing 25 μg of Hb and 10 μg of PAN was spread evenly onto the surface of pretreated GCE. The coating was dried in air at room temperature. Finally, the Hb-PAN modified electrode was immersed into phosphate buffer solution (pH 7.0) and kept in a refrigerator at 4 °C overnight to wash out the excess Hb from the electrode surface. The resultant bioelectrode was stored in a refrigerator when not to be used. The PAN-modified GCE without Hb was prepared by the same procedure.

2.3. Instrumentation

A CHI 660 electrochemical workstation was used for cyclic and amperometric voltammetry. A three-electrode cell was used with a saturated calomel electrode (SCE) as reference electrode, a platinum foil as counter electrode, and the modified glassy carbon electrode (diameter 3 mm) as working electrode. Electrolytic solutions were purged with highly purified nitrogen for at least 20 min prior to the series of experiments. A nitrogen environment was then kept over solution in the cell to prevent the contact of the solution from oxygen.

Ultraviolet–visible measurements were carried out by using a UV-2550 UV/Vis spectrophotometer. Sample films for measurements were prepared by casting Hb, PAN or Hb-PAN solution onto indium tin oxide (ITO) glass and dried in air.

Fourier transform infrared (FT-IR) spectra were recorded on Tensor 27 spectrometer. Hb solution and Hb-PAN mixture was cast on a glass, respectively. After the membrane on the glass was dried in air, it was stripped off and tabletted with KBr powder for FT-IR measurement.

3. Results and discussion

3.1. Spectroscopic analysis of PAN and Hb-PAN

UV–vis spectroscopy is an effective means for monitoring the possible change of the Soret absorption band in the heme group region. The band shift may provide information on the tertiary structure of heme proteins, especially that conformational change around the heme region [15]. Fig. 1 shows the spectra of dry Hb, PAN and Hb-PAN films on ITO glasses. For the entrapped Hb, the Soret band is located at 407 nm, shifting only 3 nm toward the red in comparison with that of dry Hb alone on ITO glass. The slight shift in Soret band and the decrease in its absorbance may be due to the interaction between PAN and proteins for the surface potential energy and adsorption. Such interactions neither destroy the structure nor change the fundamental microenvironment of the biomolecules.

The interaction between PAN and Hb could be further evaluated with the FT-IR spectra of Hb, PAN and Hb-PAN composite film. As it is well known, the shapes and positions of the amide I and amide II infrared absorbance bands of proteins provide detailed information on the secondary structure of polypeptide chain of the proteins [9,16]. The amide I band at 1700–1900 cmp^{-1} is caused by the C═O stretching vibrations of the peptide linkage. The amide II band at 1600-1500 cm^{-1} results from a combination of N–H in-plane bending and C–N stretching of the peptide groups. These two amide bands are sensitive markers for protein conformation changes. Pure Hb demonstrated an IR peak at 1650 cm^{-1} for amide I band, and a smaller one at 1535 cm^{-1} for amide II band, while for Hb-PAN, the amide I band appeared at 1660 cm^{-1} and the amide II band at 1525 cm^{-1} (Fig. 2a and c). However, in the amide II band region, PAN has no absorbance at all (Fig. 2b); the IR peak at 1525 cm^{-1} for Hb-PAN is thus attributed solely to the amide II band of Hb, which showed a very similar position to that of pure Hb. All these suggest that the protein in PAN essentially retains its native confirmation. On the other hand, the amide II band at 1535 cm^{-1} in the spectrum of pure Hb shifted to 1525 cm^{-1} in the spectrum of Hb-PAN, which might result from the weak interaction between the Hb and some specific sites of PAN.

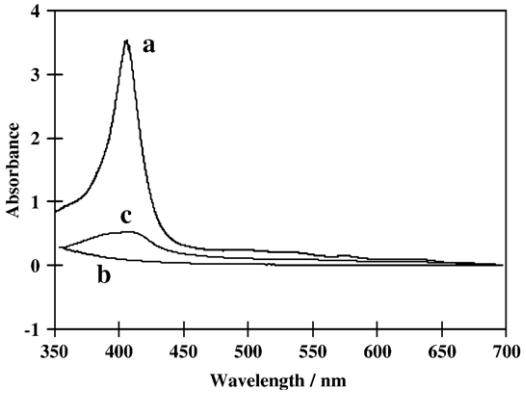

Fig. 1. UV–Vis absorption spectra of (a) Hb films, (b) PAN and (c) Hb-PAN films immobilized on ITO glass.

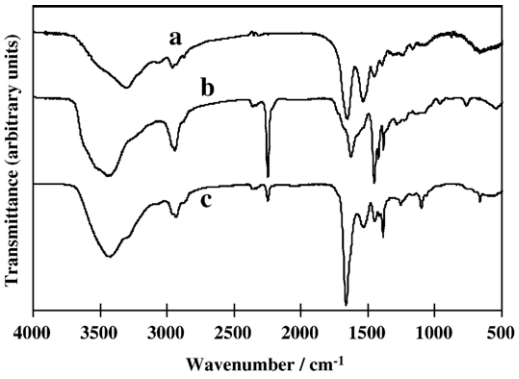

Fig. 2. FT-IR spectra for (a) Hb, (b) PAN and (c) Hb-PAN.

3.2. Direct electrochemistry of Hb-PAN modified GCE

Fig. 3 shows the cyclic voltammograms (CVs) of Hb/GCE, Hb-PAN and PAN modified GCE in deaerated 0.1 mol L^{-1} PBS (pH 7.0). A pair of well-defined redox peaks, which could be ascribed to the electron transfer between the hemoglobin and the underlying electrode, was observed at the Hb-PAN/GCE (curve c in Fig. 3). No obvious redox peak is observed at the Hb/GCE (curve b in Fig. 3), which is possibly due to the unfavorable orientation or denaturing of hemoglobin molecules on the bare glassy carbon electrode surface [17]. In addition, PAN modified GCE is electrochemically silent (curve a in Fig. 3).

The anodic peak potential (E_{pa}) and cathodic peak potential (E_{pc}) are located at −273 and −450 mV, respectively. Its formal potential (defined as the average of E_{pa} and E_{pc}), E°′ is ca. −361 mV (at 150 mV s^{-1}). The large separation between the anodic and the cathodic peak potentials ($\Delta E_p = E_{pa} - E_{pc}$ = 177 mV at 150 mV s^{-1}) suggests that the electrochemical process of Hb confined on PAN/GCE is quasi-reversible. The large peak-to-peak separation is probably due to the non-conductivity of PAN.

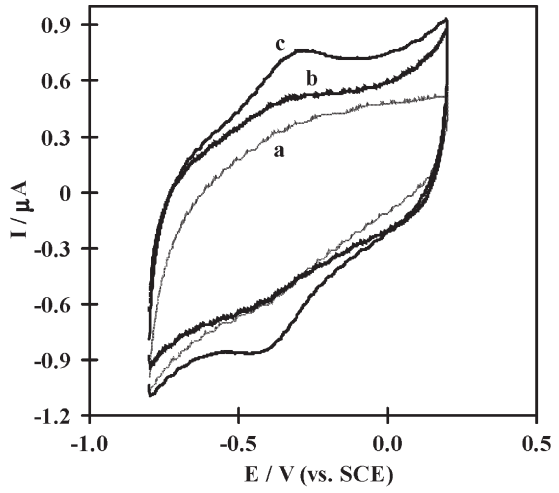

Fig. 3. Cyclic voltammograms of (a) PAN/GCE, (b) Hb/GCE and (c) Hb-PAN/GCE in 0.1 mol L^{-1} PBS (pH 7.0) at scan rate of 150 mV s^{-1}.

The average surface coverage (Γ) can be estimated according to the Laviron equation [18]

$$I_p = \frac{n^2F^2Av\Gamma}{4RT} = \frac{nFQv}{4RT} \quad (1)$$

Where Γ (mol cm^{-2}) is the surface coverage of the adsorbed Hb on the electrode surface, A is the electrode real area (cm^2), and Q is the quantity of charge integrated from the anode peak area of the voltammogram in Fig. 4A. Plotting the peak current

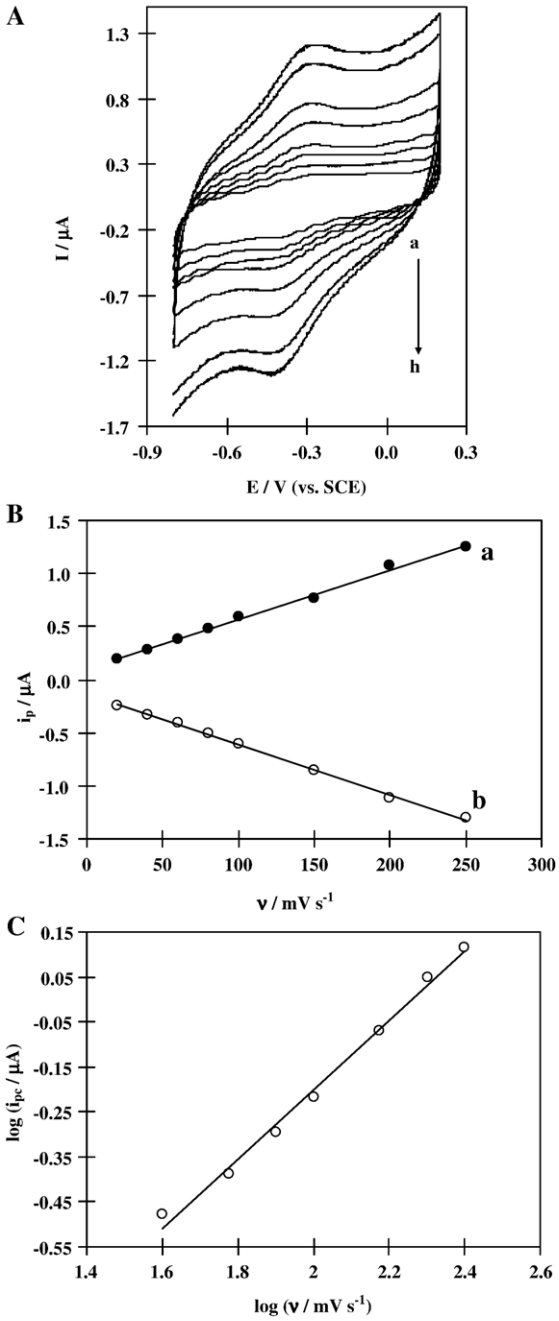

Fig. 4. (A) Cyclic voltammograms of Hb-PAN /GCE in 0.1 mol L^{-1} pH 7.0 PBS at different scan rates. The scan rates form in the inner to outer are (a) 20, (b) 40, (c) 60, (d) 80, (e) 100, (f) 150, (g) 200 and (h) 300 mV s^{-1}, respectively. (B) The plots of anodic and cathodic peak currents vs. scan rates. (C) Plot of logarithm of i_{pc} vs. logarithm of v.

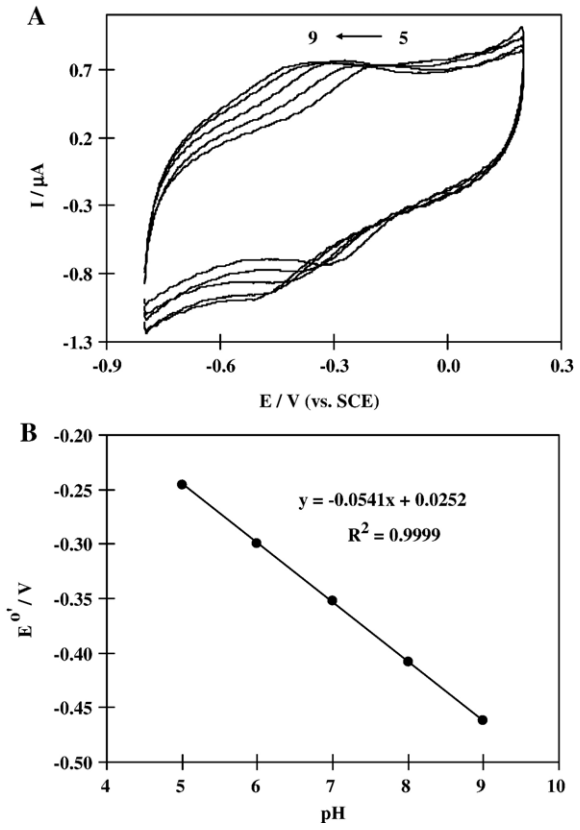

Fig. 5. (A) Cyclic voltammograms of Hb-PAN/GCE in PBS with various pH values at 150 mV s^{-1}. (B) Effect of pH on formal potential of Hb-PAN/GCE in 0.1 mol L^{-1} PBS.

I_p against the scan rate v, let us determine the surface average coverage of Hb and the number of electrons involved in the direct electron transfer on the PAN film electrode. From the slope of the I_p versus v curve, the number n of electrons involved in the direct electron transfer reaction of Hb was calculated to be $n=0.9$, based on Eq. (1), indicating that the redox reaction of the adsorbed Hb on the PAN electrode was a single electron transfer reaction. In addition, the average coverage of the adsorbed Hb on the PAN film electrode was 1.65×10^{-10} mol cm^{-2} (geometric area of the electrode was taken). The percentage of the electroactive Hb on the electrode surface was relatively low (3%), indicating that some protein in the film remain electrochemically inactive. This implies that only those Hb molecules in the inner layers of the film closest to the electrode surface are electrochemically addressable. The same phenomenon was observed with Hb-polyamidoamine dendrimer film [19]. However, the value is 9 times as large as the theoretical monolayer coverage (1.89×10^{-10} mol cm^{-10} for Hb), which was estimated to take into account the crystallographic dimensions 6.4 nm × 5.5 nm × 5.0 nm for Hb (in four heme-containing chains) [20]. Therefore, the present material PAN is promising for increasing the functional density of Hb, allowing the electric communication with the underlying GCE.

The cyclic voltammogram of Hb-PAN/GCE displays a well-defined peak shape at different scan rates and shows an almost equal height of reduction and oxidation peaks at the same scan rate (Fig. 4A). Peak currents vary linearly with the scan rates, as shown in Fig. 4B, indicating a surface-controlled electrode process. The slope obtained by linear regression of log I_{pc} versus log n is 0.77 for Hb (Fig. 4C), which corresponds to the characteristic of a thin layer electrochemical behavior.

The electron transfer rate constant k_s can be estimated with equation $k_s=mnFv/RT$ when the peak-to-peak separation was less than 200 mV [18], where m is a parameter related to the peak-to-peak separation.

The k_s value was calculated to be 1.04 s^{-1}at the scan rate of 150 mV s^{-1}, which is larger than that of Hb immobilized on a hexagonal mesoporous silica modified GCE (0.92 ± 0.18 s^{-1}) [21]. Thus, PAN can provide a microenvironment for Hb to undergo facile electron transfer reaction.

3.3. Effect of solution pH on the direct electron transfer of immobilized Hb

In most cases, the solution pH is essential to the electrochemical behaviors of proteins. Generally, the E°′ of the redox couple depends on solution pH which indicates the participation of proton in the redox process. The cyclic voltammograms of Hb-PAN/GCE at different pH solutions were measured at a scan rate of 150 mV s^{-1} (Fig. 5A). An increase of solution pH from pH 5.0 to 9.0 led to a negative shift of both oxidation and reduction peak potentials. The formal potential is found to be linearly decreased with increasing pH from 5.0–9.0 with a slope of −54 mV pH^{-1} (Fig. 5B.). This value is approximately close to the theoretical value of −57.6 mV pH^{-1} for a reversible proton-coupled single electron transfer [22]. This suggests that a single protonation accompanies electron transfer between the electrode and each of the four heme Fe(III) of Hb, represented in general terms by

$$\mathrm{HbhemeFE(III)} + \mathrm{H^+} + \mathrm{e^-} \Leftrightarrow \mathrm{HbhemeFe(II)}.$$

where the charges on Hb species have been omitted.

3.4. Electrocatalysis of the Hb-PAN/GCE toward H_2O_2

Hemoglobin has certain intrinsic peroxidase activity due to its close similarity with peroxidase, so it can be employed to catalyze

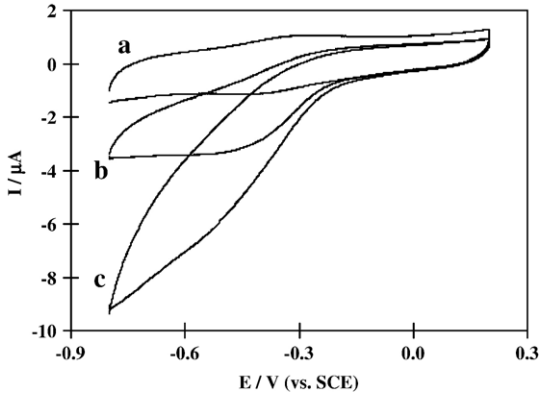

Fig. 6. Cyclic voltammograms of Hb-PAN/GCE in 0.1 mol L^{-1} pH 7.0 PBS (a) and in the presence of 0.1 mmol L^{-1} H_2O_2 (b) and 1.6 mmol L^{-1} H_2O_2. (c) Scan rate: 150 mV s^{-1}.

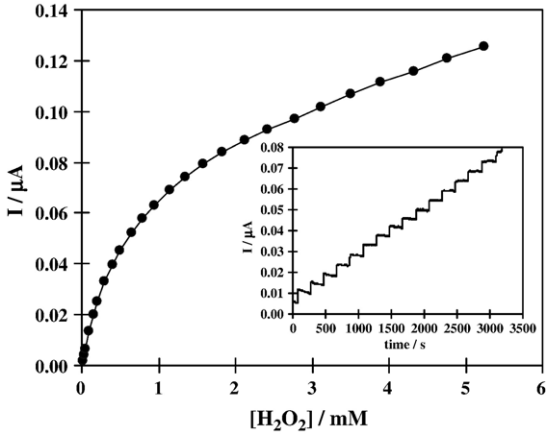

Fig. 7. Calibration curve of Hb-PAN/GCE for H_2O_2. Inset: Typical steady-state response of the Hb-PAN /GCE on successive injection of H_2O_2 into stirring 0.1 mol L^{-1} PBS. Applied potential: −250 mV.

the reduction of H_2O_2. Electrocatalytic reduction of H_2O_2 using Hb-PAN/GCE was investigated by CVs. When H_2O_2 was added to a pH 7.0 buffer solution, an increase in the reduction peak was observed with the decrease of the oxidation peak for Hb (Fig. 6, curve b and c). The reduction peak current increases with the concentration of H_2O_2 in the solution, indicating that the immobilized Hb exhibits excellent electrocatalytic activity towards the reduction of H_2O_2.

The electrocatalytic reduction of hydrogen peroxide at Hb-PAN/GCE was also studied by amperometry, which is one of the most widely employed techniques for biosensor. Fig. 7 shows a typical calibration curve of the Hb-PAN/GCE for H_2O_2 determination. The applied potential is controlled at −0.25 V (vs. SCE). The inset of Fig. 7 displays the successive injection of H_2O_2 at optimized conditions on successive injections of H_2O_2 to stirring PBS at pH 7.0. A clearly defined reduction current proportional to the hydrogen peroxide concentration was observed. The biosensor achieved 95% of the steady-state current within 5 s, with a linear range of 8.3×10^{-6} to 5×10^{-4} mol L^{-1}. The detection limit was 8.3×10^{-6} mol L^{-1} based on $S/N=3$.

The apparent Michaelis–Menten constant (K_M^{app}) can be obtained by the analysis of the slope and the intercept of the plot of the reciprocals of the steady-state current versus H_2O_2 concentration. K_M^{app} value for Hb-PAN film modified GCE was found to be 0.9 mmol L^{-1}, which implies that the present electrode exhibits a high affinity to H_2O_2.

4. Conclusion

PAN has been demonstrated as an attractive material to immobilize Hb to achieve its direct electron transfer on GCE. The results of UV–Vis and FT-IR indicated that the protein in PAN retained near-native secondary structures. Redox peak currents increased linearly with the increase of scan rate, indicating a surface-controlled electrode process. Stable protein/PAN-modified electrodes can be used to determine hydrogen peroxide. This material can provide a favorable microenvironment for redox proteins and enzymes, and thus it is expected to have widely potential applications in direct electrochemistry, biosensors and biocatalysis.

Acknowledgments

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