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Direct electrochemistry of hemoglobin in the hyaluronic acid films

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Abstract

Hemoglobin (Hb) in the hyaluronic acid (HA) was cast at pyrolytic graphite (PG) electrodes for researching its electrochemical and electrocatalytic properties. The formal potential and electron transfer rate constant of Hb on HA films were determined, and the stability of the films, the pH effect, and the influence of supporting electrolyte concentrations upon Hb electrochemistry on the films were investigated by cyclic voltammetry and square wave voltammetry. UV–Vis absorption and reflectance absorption infrared (RAIR) spectra showed that the protein on HA film retained near-native secondary structure. The stable Hb–HA/PG gave analytically useful electrochemical catalytic responses to hydrogen peroxide. Thus, the property of the HA film for sorption and retention of water maybe utilized to develop some new biosensors.

Keywords: Hemoglobin; HA; Redox chemistry; Enzymatic; Catalysis

1. Introduction

Hemoglobin (Hb), a tetramer protein with an M_r of approximately 67 kDa, is composed of 4 subunits, namely $\alpha_2\beta_2$. It contains 4 electroactive iron hemes as its prosthetic groups, with one heme per subunit. Although Hb itself does not physiologically function as an electron carrier, still it is an ideal model for the study of electron transfer reactions of heme proteins.

During the recent years, studies of direct electrochemistry of proteins or enzymes have been reported. A good method of researching on the electrochemistry of proteins is to incorporate proteins into films modified on the surface of electrode [1]. Thin film may provide a favorable microenvironment for the proteins and can be used to enhance the electron transfer between the proteins and electrodes. Rusling incorporated Myoglobin (Mb) and Hb into didodecyldimethylammonium bromide (DDAB) surfactant films on the edge-plane pyrolytic graphite (PG) electrodes and the electron transfer rates were remarkably improved [2,3]. Hu et al. reported that direct electron transfer rates were increased greatly for Mb, Hb and horseradish peroxidase (HRP) in a series lamellar liquid crystal surfactant films, such as hydrogel polymers, protein–clay films, and polyelectrolyte–surfactant on basel-plane pyrolytic graphite (PG) [4–9]. These modified films provide a suitable microenvironment for the proteins in the films and are helpful for electrochemical studies of the proteins. In addition, a kind of new film was also applied to coat the surface of the pyrolytic graphite electrode to increase the direct reversible electron transfer. Hu had made use of bilayer membranes similar to biological membranes, formed by lipids in living organisms [10–15]. These works also facilitated the researches on fabricating biosensors, enzymatic bioreactors, and biomedical devices. Thus, the film's function in direct electron exchange between protein and electrode is significant.

Hyaluronic acid (HA) consists of 2-acetamide-2-deoxy-α-D-glucose and β-D-glucuronic acid residues linked by alternate (1 → 3) and (1 → 4) glycoside bonding. Its chemical structure HA is shown in Fig. 1. HA's utilization was mainly based on its pile-screwing conformation, which had lots of hydroxyl on the inside-wall. As a component of the glycosaminoglycans (GAGs) inextracellular matrix (ECMs), HA has a high capacity for the sorption and retention of water and effects several cellular functions such as adhesion, migration, and proliferation [16,17]. The biomedical applications of HA include scaffolds for wound healing and tissue engineering [18,19], as well as ophthalmic surgery, arthritis treatment, which demonstrated that HA had a good biocompatibility. Therefore, hyaluronic

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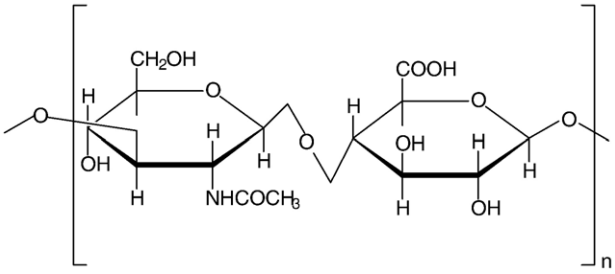

Fig. 1. The chemical structure of HA.

acid can be utilized as a film to study the electron transfer of proteins on electrodes.

For the purpose of fabricating protein–film structures, it would be more advantageous to use polysaccharides, biodegradable and environmentally benign materials as film-forming materials than synthetic polymers. We think that HA could form stable films on the edge-plane PG electrodes in aqueous solutions, and redox proteins incorporated into the films would show good electrochemical properties. To our knowledge, only the electrochemistry properties of Mb proteins have been studied by Hu on a basal-plane pyrolytic electrode modified HA film [20].

In present paper, we used the simple cast method to immobilize Hb into HA films on the edge-plane PG electrodes and studied Hb–HA films by UV–Vis spectroscopy and investigated its catalytic properties.

2. Materials and methods

2.1. Materials

Bovine hemoglobin (Hb, M_r 67 kDa) was from Sigma. Hyaluronic acid (HA, M_r 1500 kDa) was a gift from University of Beijing Chemical Technology. A buffer of phosphate was used in 1×10^{-2} M (pH=4–9) containing 50 mM NaBr that as supporting electrolyte. The pH values were adjusted with HCl or KOH solutions. Hemoglobin was dissolved in buffers of 10 mM phosphate, pH 5.5. The concentration of Hb was determined by UV–Vis spectroscopy using å_{406}=41 000 M^{-1} cm^{-1} [21]. All other chemicals were reagent grade. Water was purified by successive ion exchange and distillation. The working electrodes (edge-plane pyrolytic graphite (EPG)) were constructed from pyrolytic graphite (Advanced Ceramics Corp. Europe) being perpendicular to the a–b plane and housed in Teflon sheaths.

2.2. Film preparation

Edge-plane PG electrode was polished to mirror smoothness on polishing cloth with 0.3 μm and 0.05 μm alumina, respectively. Then, the electrode was ultrasonicated in deionized water for 1 min. 1×10^{-3} M Hb in buffer pH5.5 was mixed with 1 mg/mL HA solutions at pH 5.5 (containing 0.05 mol/L NaBr) in a 1:1 (v/v) ratio. 20 μL of this mixture was spread uniformly onto an edge-plane PG electrode, then, dried in the air.

2.3. Apparatus

Cyclic voltammetry (CV) and square wave voltammetry (SWV) were carried out with a computer-controlled electrochemical workstation (CHI 660b, Shanghai Chenhua, China), a three-electrode cell with a saturated calomel reference electrode (SCE), a platinum auxiliary electrode and an edge-plane pyrolytic graphite (PG) with the disk of geometric area 0.12 cm^2 as the working electrode.

UV–visible spectrophotometer (HP8453) was used to measure the concentrations of Hb solutions and sample films which were prepared by depositing protein–HA solutions onto quartz glass slides, and then dried in the air. IR spectra were obtained by using IFS-66/S spectrophotometer.

2.4. Cyclic voltammetry

Edge-plane PG electrodes coated with Hb–HA films were placed into 10 mM phosphate buffer, pH 7.0. Since protein stability and conformation could be affected by salt as well as pH, NaBr was maintained at 5×10^{-2} M in all buffers. Experiments were carried out at 20±0.1 °C. The solutions were purged with purified nitrogen for 20 min to remove oxygen prior to each experiment. A nitrogen environment was then kept in the cell with a continuously N_2 flow during the whole experiment. All potentials were referred to the SCE.

3. Results

3.1. Cyclic voltammetry

No CV peaks were observed on a bare edge-plane PG or an HA/EPG electrode in 0.1 M hemoglobin (Hb) solution in pH 7.0 phosphates buffer. It showed that electron transfer between these electrode and Hb in this solution occurred very slowly or even not at all [22]. However, when an edge-plane PG electrode coated with Hb–HA film immersed into the 1×10^{-2} M buffers without Hb (pH 7.0), a pair of well-defined and reversible redox

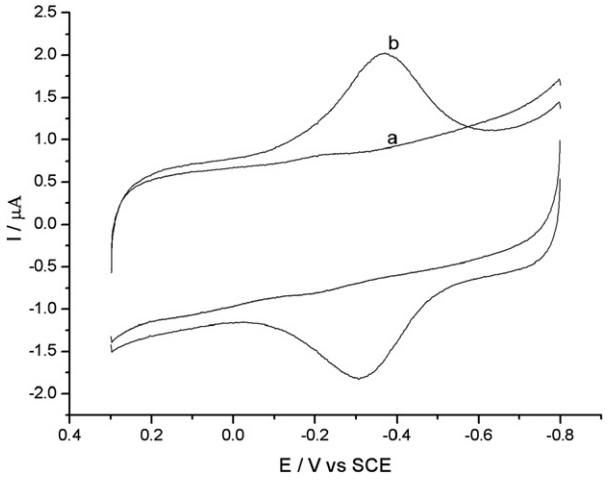

Fig. 2. Cyclic voltammograms at 0.1 V s^{-1} in pH 7.0 buffers without Hb: (a) HA films, and (b) Hb–HA films.

peaks were observed (Fig. 2b). The anodic peak potential was found to be $E_{pa}=-0.31$ V and the cathodic peak potential to be $E_{pc}=-0.37$ V at a scan rate of 0.1 V s^{-1}. The formal potential $E^{\circ\prime}$ was −0.34 V (vs. SCE), which is characteristic of the Hb heme Fe (III)/Fe (II) redox couple. In contrast, HA films without Hb showed no CV peaks at all in this potential window (Fig. 2a). So, HA films should have a great effect on the kinetics of the electrode reaction for the proteins to transfer electrons with underlying electrodes.

Cyclic voltammograms of Hb–HA/EPG showed nearly symmetric anodic and cathodic peaks and roughly equal height. The reduction and oxidation peak currents increased linearly with scan rate from 0.02 to 2 V s^{-1} (Fig. 3). The anodic to the cathodic peak potential difference E_p is 64 mV at 0.1 V s^{-1}. These results are characteristic of quasi-reversible, surface-confined electrochemical behavior, in which all electroactive proteins in their heme Fe (III) forms in the films are reduced on the forward cathodic scan, and the reduced proteins in their heme Fe (II) forms are then fully oxidized to the heme Fe (III) forms on the reversed anodic scan.

Since a long-term stability is one of the most important properties required for a biosensor or bioreactor, the stability of the Hb–HA film electrode has being also investigated by CVs. The electrode with Hb–HA films was stored in the air and the films were retained dry. CVs were run occasionally in the buffers. The Hb–HA films were so excellent stability that the Hb–HA film electrode exhibited no changes in CV peak potential and the peak height decreased only 16% compared with the initial steady state value in 30 days.

To estimate the apparent heterogeneous electron transfer rate constant (k_s), square wave voltammetry (SWV) and non-linear regression methods were applied to the Hb–HA films [23,24]. SWV data can be easily fitted to get the kinetic parameters. Examples for Hb–HA films are showed in Fig. 4. The average k_s and $E^{\circ\prime}$ values for Hb films at pH 7.0 are estimated to be 5.19 s^{-1} and −0.36 V, respectively. Though the value of the electron transfer rate is small, the response of the Hb–HA films

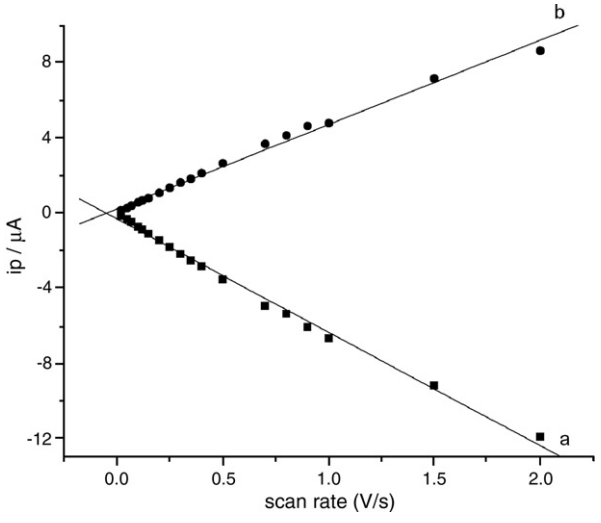

Fig. 3. The relationship of the current peak with scan rate for Hb–HA films at pH 7.0 buffers: (a) the anodic peak current, and (b) the cathodic peak current.

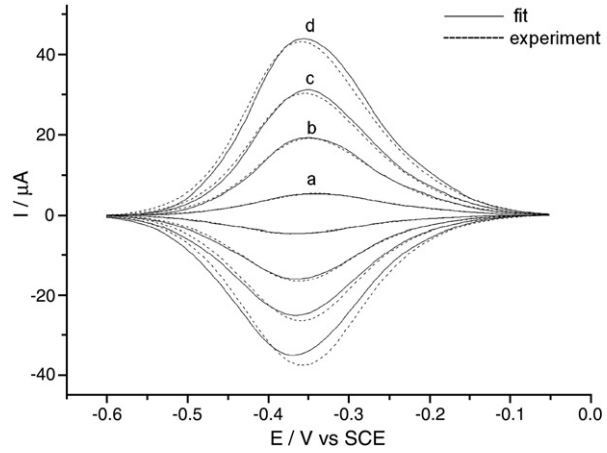

Fig. 4. Square wave forward and reverse current voltammograms for Hb–HA films in pH 7.0 buffer solutions at different frequencies. The dashed lines represent the experimental SWVs from which the background has been subtracted. The solid lines are the best fit by nonlinear regression onto the 5-$E^{\circ\prime}$ dispersion model. SWV conditions: pulse height 60 mV; step height 4 mV, and frequencies (Hz): (a) 15; (b) 25; (c) 30; (d) 35.

to the edge-plane PG electrode is enhanced greatly. The reason may attribute to the electrostatic attraction of the positively charged hemoglobin at pH 5.5 and the negatively charged hyaluronic acid, thus Hb is successfully attracted by the driving force, and then is absorbed onto the negatively charged edge-plane PG electrode. The favorable orientations of protein at electrodes maybe lead to electron transferring easy. So the Hb–HA films must have a great effect on the kinetics of the electrode reaction for the protein to transfer electrons with the underlying electrode.

3.2. pH effect

Nearly reversible voltammograms with stable and well-defined redox peaks were obtained in the pH range of 5.0–9.0. As the pH increases, $E^{\circ\prime}$ shifts negatively. The shift in $E^{\circ\prime}$ depended on pH suggested that the redox reaction was accompanied by the transfer of protons. The linear regression equation

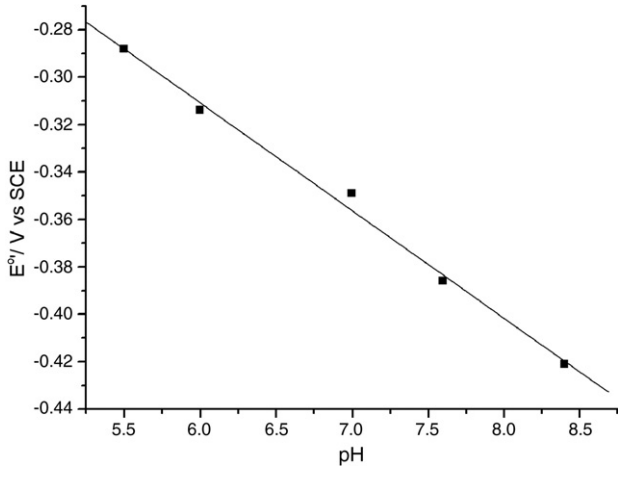

Fig. 5. Influence of pH on the potentials estimated by CV at 0.1 V s^{-1} for Hb–HA films on the edge-plane PG electrodes.

of E (V) = −0.038–0.045 pH with a correlation coefficient of 0.99 (n = 5) (Fig. 5) was obtained. The slope value of 45 mV/pH was smaller than the theoretically expected value of 59 mV/pH [25], which suggested that one proton transfer was coupled to one electron transfer for each heme group of Hb during the reversible electrode reaction. The reaction represented in general terms by

$$\mathrm{HbhemeFe^{III} + H^+ + e^- = HbhemeFe^{II}}. \quad (1)$$

Why the slope value became to be 45 mV/pH? We think that it may be due to the influence of the protonation states of trans-ligands to the heme iron and amino acids around the heme, or the protonation of the water molecule coordinated to the central iron ion [26].

3.3. Effect of ionic strength

NaBr was used abroadly as supporting electrolyte, its concentration changes had an influence on heterogeneous electron transfer rate [27]. When the concentration of NaBr as supporting electrolyte in phosphate buffers changed, the CVs showed the unchanged peak potentials but changed current height. As can be seen from Fig. 6, when PBS buffers were 1×10^{-2} M, and the concentration of NaBr (C_{NaBr}) is changed from 0 to 1×10^{-1} M, the anodic peaks current i_a is linearly dependent on C_{NaBr}. This suggests that the supporting electrolyte has an influence on peak current, more specifically, the rate of electron transfer between the protein and the electrode enhances with the increased concentration of supporting electrolyte.

3.4. Spectroscopy

3.4.1. Reflectance Absorption Infrared (RAIR) spectroscopy

RAIR is a surface infrared technique, which is ideally suited for exploring the secondary structure of protein on a solid substrate surface. The retention of the Hb conformation was further verified by reflectance FT-IR measurement. As shown in

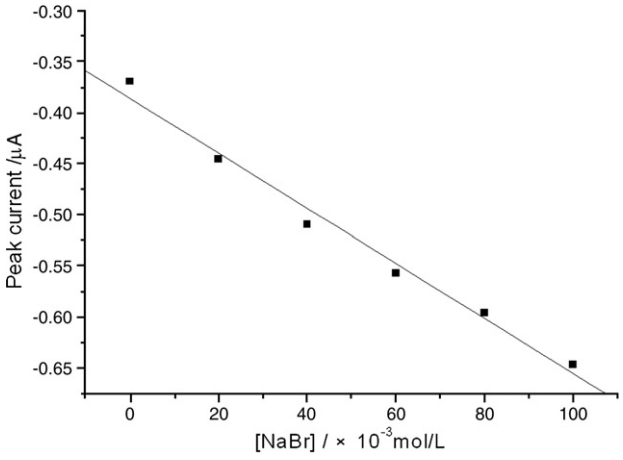

Fig. 6. Influence of ionic strength on the peak current estimated by CV at 0.1 V s^{-1} for Hb–HA films on the edge-plane PG electrode.

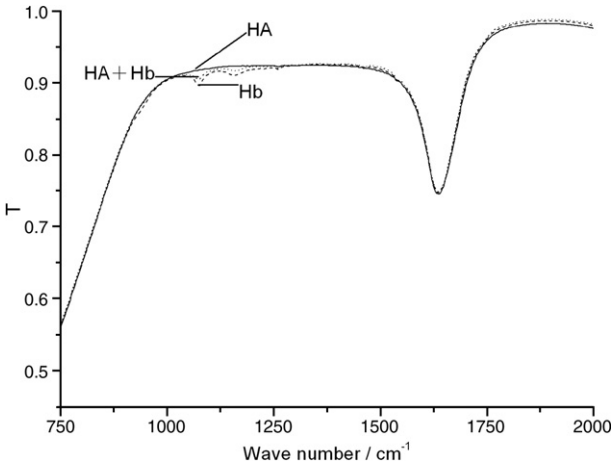

Fig. 7. RAIR spectra of Hb, HA and Hb–HA films on the EPG electrode surfaces.

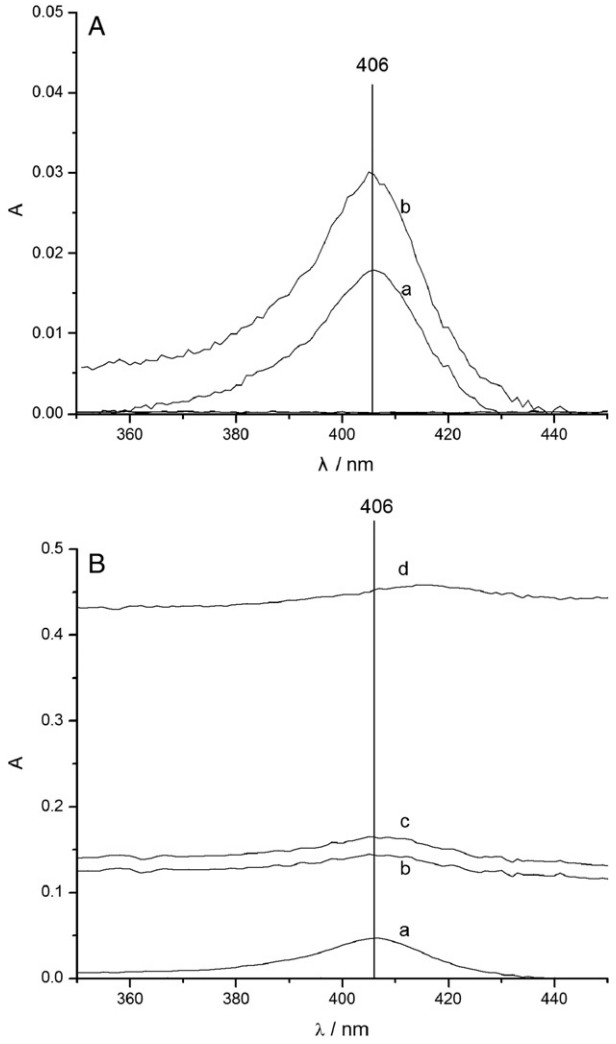

Fig. 8. (A) UV–Vis spectra of Hb (a) and Hb–HA (b) films on quartz glass slides; (B) UV–Vis spectra of Hb–HA films on quartz glass slides with drying of the Hb–HA films from a to d.

Fig. 7, the shape and the position of amide (1600–1700 cm^{-1}) infrared bands provide detailed information for the secondary structure of polypeptide chain of proteins [28,29]. The shapes of amide bands in the RAIR spectra of Hb in HA film are similar to that of the Hb film alone, which supports the viewpoint that Hb entrapped in HA film retains a secondary structure similar to the native form.

3.4.2. UV–Vis absorption spectroscopy

Position of the iron heme Soret absorption band of Hb could provide the information about possible denaturation of protein. Fig. 8A shows that Hb in HA films spreading on the quartz glass with buffers has an absorption band at the same wave of 406 nm as Hb alone, indicating that the secondary structure of Hb in the HA films is similar to the native structure of Hb, and the conformation of Hb in the HA films does not change in buffers.

When films were becoming dry, the Soret bands of Hb were observed to shift to red direction (Fig. 8B) clearly. The Soret bands shift from 406 nm (a, b, c) to 415 nm (d). However, when the buffer solution was added onto the dried films, the Soret bands shifted back to 406 nm (a) again. This small shift proved that Hb keeps its original structure in HA film whatever the Hb–HA films is in solution or dry. We believe that this is the first observation so far.

3.5. Catalytic reactivity

The electrocatalytic behavior of the protein–HA films towards hydrogen peroxide were tested by CV. When H_2O_2 was added to the buffers, there was no peak on the HA film electrode at all. However, when H_2O_2 was added to the buffers, the HbFe III reduction peak on the Hb–HA film electrodes increased in height at approximately −0.34 V (Fig. 9), accompanied by a decrease and disappearance of the HbFe II oxidation peak. Moreover, the reduction peak current increased with an increase of H_2O_2 in solution. The direct reduction of H_2O_2 on blank HA films was not observed in the determining range of potential range, so these results were consistent with the reduction of H_2O_2 by HbFe II in a catalytic cycle.

Catalytic reduction of H_2O_2 also proved that the characteristic of the protein in the HA films on the edge-plane electrode did not change. The protein in HA films kept its catalytic active in the buffer solutions.

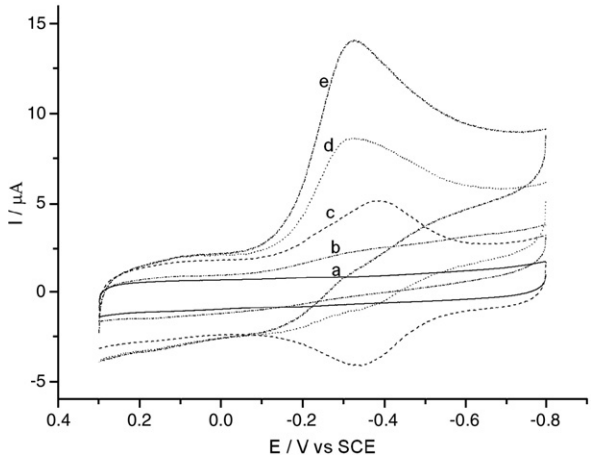

Fig. 9. Cyclic voltammograms at 0.3 V s^{-1} in pH 7.0 buffers for (a) HA films with no H_2O_2 present, (b) HA films with 8×10^{-5} M H_2O_2 present, (c) Hb–HA films with no H_2O_2 present, (d) Hb–HA films with 8×10^{-5} M H_2O_2 present, (e) Hb–HA films with 1.2×10^{-4} M H_2O_2 present.

4. Conclusions

This work investigated the direct heterogeneous electron transfer reaction for Hb in HA films at edge-plane PG electrode. The positively charged Hb at pH 5.5 with the negatively charged hyaluronic acid was successfully adsorbed onto the electrode, indicating that the main driving force for the Hb with HA should be electrostatic attraction. Visible absorption and reflectance–absorption infrared spectra proved that the Hb entrapped in HA was in its native status and Hb retains its near native structure in Hb–HA films in dry form and in the medium pH range. The catalytic reaction proved that Hb in HA films also remained their electroactivity.

5. Simplified description of the method and its (future) applications

The hemoglobin protein entrapped in the HA film exhibited its stable and reversible electrochemistry characters. The structure and properties of the protein in the film did not change, and the response of the protein in electrochemistry also enhanced. In addition, as biodegradable and environmentally benign materials, polysaccharides would be more advantageous than synthetic polymers when it is used as film-forming materials for fabricating protein–film structures. Thus, the property of sorption and retention of water in the HA film can be utilized in aqueous solution system.

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