Electrochemical studies on polysorbate-20 (Tween 20)-entrapped haemoglobin and its application in a hydrogen peroxide biosensor

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Haemoglobin (Hb) was entrapped in polysorbate 20 and then modified on a pyrolytic graphite electrode. Electrochemical studies revealed that a pair of stable and well-defined redox peaks attributed to the direct redox reaction of Hb could be observed in a phosphate buffer solution (pH 6.0). The anodic and cathodic peaks were located at −236 and −316 mV (versus a saturated calomel reference electrode) separately. The formal potential, $E^{0\prime}$, was linearly varied with pH in the range from 3.0 to 10.0 with a slope of $-48.0\ mV \cdot pH^{-1}$. Moreover, the protein was capable of catalysing the reduction of H_2O_2. Accordingly, an unmediated biosensor for H_2O_2 was prepared with a linear range from 8.0×10^{-7} to 1.0×10^{-3} M. This biosensor exhibited good stability, sensitivity and reproducibility.

Introduction

Protein electrochemistry has attracted more and more interest from the viewpoint of both understanding the fundamental features of *in vivo* systems and applications such as the construction of biosensors. However, it is well known that, in most cases, it is difficult for proteins to exhibit heterogeneous electron-transfer processes [1,2]. Recent developments in protein film voltammetry afford new and as yet largely unexploited opportunities for studying the redox properties of proteins [3–8]. In this method, proteins are entrapped in monolayers or multilayers on electrode surfaces, so that diffusion of these large species to electrodes is not a limiting factor. The high local concentration of protein sequestered on the electrode surface can give much larger and better-defined voltammetric peaks compared with the same proteins in solution. So far, many kinds of materials have been reported to conduct direct electron-transfer reactions of haem proteins [9–20]. All these films clearly enhance the direct electron transfer between proteins and electrodes compared with that on bare electrodes with the proteins in solution.

Determination of H_2O_2 is of particular importance in clinical, environmental and many other fields. Haemoglobin (Hb) has some intrinsic peroxidase activity due to its close structural similarity to peroxidases [21]. Therefore it can be employed to catalyse the reduction of H_2O_2 and to develop unmediated biosensors for H_2O_2. In the present study, we report the first attempt to immobilize Hb in polysorbate 20 (a non-ionic surfactant, also generally known as Tween 20) by forming a mixture of the aqueous vesicle of polysorbate 20 and Hb on PG (pyrolytic graphite) electrodes. Experimental results reveal that Hb can exhibit direct electrochemical behaviour in polysorbate 20 films. Moreover, it can retain its bioactivity in the polymer films and exhibit excellent catalytic activity towards H_2O_2. An H_2O_2 biosensor was thus developed.

Experimental

Chemicals

Human Hb (molecular mass 66 kDa) and polysorbate 20 (880 Da) were obtained from Sigma. They were used without further purification. Other chemicals were of analytical grade. All solutions were prepared in double-distilled water, which was purified with a Milli-Q purification system (Branstead, Boston, MA, U.S.A.) to a specific resistance of $>16\ M\Omega \cdot cm^{-1}$ and stored in the refrigerator at 4°C.

Preparation of Hb-polysorbate 20 films

The PG rod was purchased from Shanghai Carbon Co. (Shanghai, People's Republic of China). The substrate PG electrode was prepared by inserting a PG rod in a glass tube and fixing it with epoxy resin. Electrical contact was made by attaching a copper wire to the rod with the help of Wood's alloy (a fusible bismuth-based alloy).

Before coating, the PG electrode was first polished using rough and fine sand papers. Its surface was then polished to mirror smoothness with alumina (particle size

Key words: cyclic voltammetry, electrocatalysis, haemoglobin (Hb), hydrogen peroxide (H_2O_2), polysorbate 20 (Tween 20), protein film voltammetry.

Abbreviations used: CV, cyclic voltammogram; PG, pyrolytic graphite; SCE, saturated calomel reference electrode.

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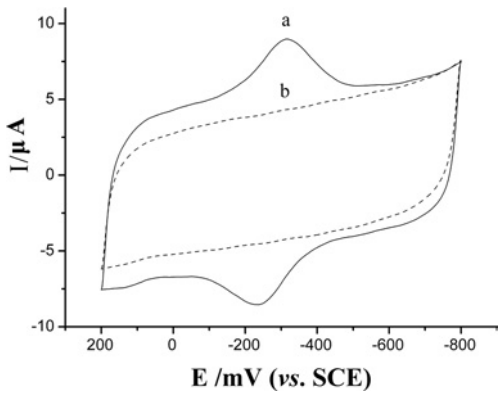

Figure 1 CVs at 200 mV · s^{-1} in a 0.1 M phosphate buffer (pH 6.0) for (trace a) a Hb-polysorbate 20 film and (trace b) a polysorbate 20 film

of approx. 0.05 μm)/water slurry on silk. Eventually, the electrode was thoroughly washed by ultrasonicating in both double-distilled water and ethanol for approx. 5 min.

To obtain the best CV (cyclic voltammogram) of Hb-polysorbate 20 films, the experimental conditions for film casting, such as the concentration of Hb, the Hb/polysorbate 20 ratio, and the total volume of Hb-polysorbate 20 dispersion, were optimized. Typically, 10 μl of the dispersion containing 12 μM Hb and 10 mg · ml^{-1} polysorbate 20 was spread evenly on to PG electrodes for preparing Hb-polysorbate 20 films. A small bottle was fitted over the electrode so that water evaporated slowly and more uniform films were formed. Films were then dried overnight in air.

Measurements

Electrochemical experiments were performed with a Potentiostat/Galvanostat 283 (PARC, EG&G, Princeton, NJ, U.S.A.) and a three-electrode system. A one-compartment glass cell with a modified PG working electrode, an SCE (saturated calomel reference electrode) and a platinum wire auxiliary electrode were used for the measurements with a working volume of 5 ml. Electrochemical experiments were performed at ambient temperature (22 ± 2 °C). The Hb–polysorbate 20 film electrodes were stored in a phosphate buffer (pH 6.0) in the refrigerator at 4 °C when they were not being used. Buffers were purged with purified nitrogen for 10 min before a series of experiments. A nitrogen environment was maintained in the cell. All the following potentials reported in this work are versus SCE.

Results and discussion

In Figure 1, traces a and b are the CVs of the PG electrodes coated with Hb-polysorbate 20 or polysorbate 20 alone respectively in a phosphate buffer solution (pH 6.0) at scan rate of 200 mV · s^{-1}. A pair of stable and well-defined redox peaks can be obtained at the Hb–polysorbate 20 coated PG electrode (Figure 1, a). The anodic and cathodic peaks occur at −236 and −316 mV (versus SCE) respectively. The formal potential, $E^{0\prime}$, calculated according to half-wave peak potential is −276 mV, which is in close agreement with previous reports [22,23]. In contrast, no corresponding wave is observed when using an electrode coated with polysorbate 20 alone (free of Hb) (Figure 1, trace b). Therefore polysorbate 20 by itself is not electroactive over the potential range of interest, but it may provide a desirable microenvironment for electron transfer between Hb and the electrode.

Both the anodic and cathodic peak currents of Hb are found to increase linearly with scan rate from 20 to 1000 mV · s^{-1} (Figure 2), which is a characteristic of thin-layer electrochemical behaviour [24], namely, nearly all electroactive met-Hb (oxidized form of Hb) on the electrode surface is converted into ferrous Hb (reduced form of Hb) on the forward CV scan and vice versa.

The effect of pH on the electrochemical behaviour of the protein was examined. Voltammetric peaks of Hb in a polysorbate 20 film shift negatively with increasing pH. The pH-induced variations in the peak shape and potentials in voltammograms are reversible, i.e. the same CV can be obtained if the Hb–polysorbate-20-modified electrode is transferred from a background solution with a different pH value to its original solution. Plots of $E^{0\prime}$ versus pH are linear with a slope of approx. −48 mV · pH^{-1} (Figure 3). This result suggests that one-proton transfer is coupled with one-electron transfer [25].

The electrocatalytic reduction of H_2O_2 by Hb immobilized in the polysorbate 20 film has been tested. Figure 4 shows the CVs of Hb–polysorbate-20-modified electrode in a phosphate buffer solution (pH 6.0) with and without 2.0×10^{-4} M H_2O_2 at a scan rate of 200 mV · s^{-1} in the potential range from −200 to 800 mV. In the presence of 200 μM H_2O_2, the cathodic peak at −280 mV increases significantly and the anodic peak almost disappears (Figure 4b), which indicates a typical H_2O_2 electrocatalytic reduction process [26]. With the concentration of H_2O_2 increasing, the cathodic peak current increases gradually. Noticeably no corresponding electrochemical signal was observed employing either a bare PG electrode or a PG electrode coated with polysorbate 20 alone (free of protein) in the same H_2O_2 solution. The catalytic reduction of H_2O_2 is therefore due to Hb.

Further experimental results reveal that the Hb–polysorbate-20-film-modified electrode will show the best response to H_2O_2 at pH 6.0. With the optimum condition, a linear dependence of the catalytic peak current on the concentration of H_2O_2 is observed over the range of 8.0×10^{-7} to 1.0×10^{-3} M (Figure 5). The linear regression equation is $y = 7.44252 + 0.03454x$, with a correlation

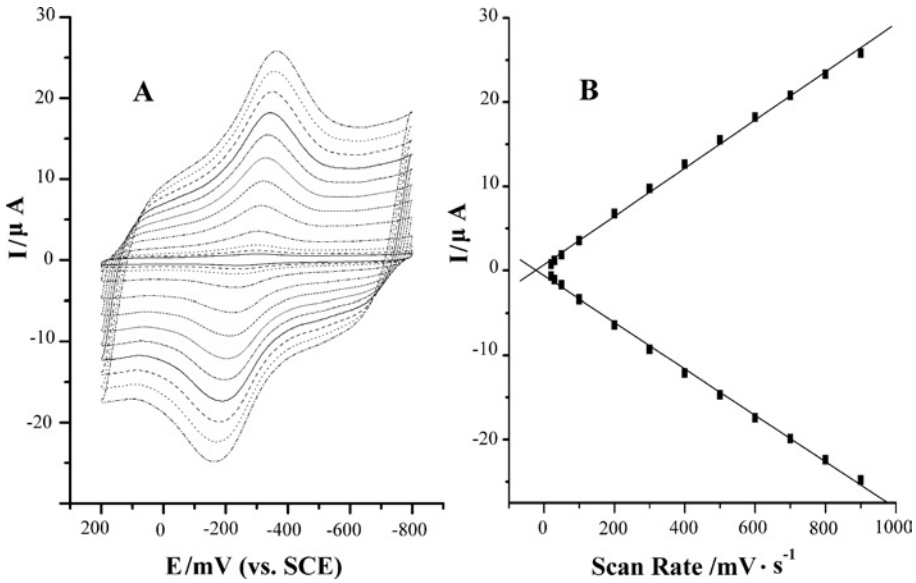

Figure 2 Relationship between peak currents and scan rate

(**A**) A series of CVs obtained at an Hb–polysorbate-20-modified PG electrode in a 0.1 M phosphate buffer solution (pH 6.0). Scan rate from 20 to 1000 mV · s^{-1}. (**B**) The redox peak currents increase proportionally with scan rate in this range.

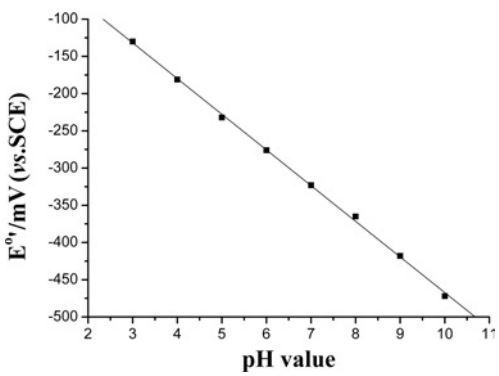

Figure 3 Effect of pH value on the formal potential ($E^{0'}$) of Hb

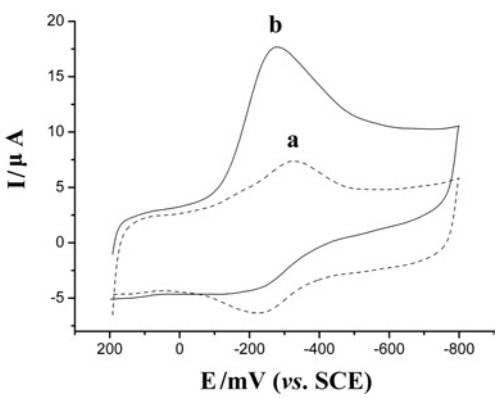

Figure 4 CVs obtained at an Hb–polysorbate-20-modified PG electrode for a 0.1 M phosphate buffer solution (pH 6.0), (a) before and (b) after the addition of 0.2 mM H_2O_2 to the buffer solution

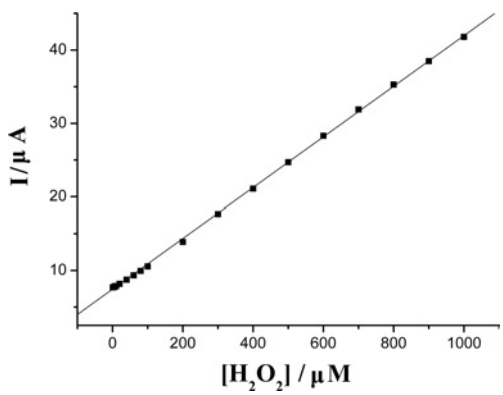

Figure 5 Linear fitting program of cathodic peak current of Hb with H_2O_2 concentration from 0.8 μM to 1 mM

coefficient of 0.9998. Compared with other methods for sensing H_2O_2, such as electrostatic layer-by-layer adsorption of horseradish peroxidase and polycation-bearing Os complex {[Os (bpy) 2Cl]$^{+/2+}$, where bpy is 2,2′-dipyridyl and Os is osmium [27]} or immobilization of Hb into egg-phosphatidylcholine films [28], the detection limit of this biosensor is lowered by approx. 10-fold. In addition, this biosensor has a larger linear range compared with the previous reports [29–31].

The reproducibility of the sensor has also been evaluated in the presence of 100 μM H_2O_2 with the same enzyme electrode, and the relative standard deviation is 2.8% ($n = 5$). The voltammetric response to 100 μM H_2O_2 decreased by <10% after 2 weeks, which indicates a good

stability. The biosensor should be stored in a refrigerator at 4°C in a phosphate buffer (pH 6.0).

We have studied five kinds of possible interfering compounds: ascorbic acid, dopamine, catechol, uric acid and adrenaline. These interfering compounds at a concentration of 0.5 mM do not interfere in the detection of 100 μM H_2O_2. However, oxygen can generate a relatively large interference in the detection of H_2O_2. Thus this kind of biosensor should be used under anaerobic conditions.

Acknowledgments

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