1. INTRODUCTION

Bioelectrochemistry is a well-established discipline both for investigating protein redox properties and for fabricating new kinds of electrochemical biosensors [1, 2]. A key factor for successful bioelectrochemical investigations of redox proteins is to obtain an efficient electrochemical response at some electrode surface. Incorporation of proteins into films is a relatively new approach to realize this response, which may also help to avoid contributions to the experimental data deriving from protein diffusion in the solution. So far, many kinds of films containing heme proteins have been reported to produce quite stable electroactive arrays and well-defined redox peaks [3–15]. Proteins retain the native conformations in these films, and the electron transfer rates are greatly enhanced.

Lipopolysaccharide is a primary constituent of the outer cellular membrane of gram-negative bacteria [16]. The structure of LPS varies among bacterial species but is overall composed of three parts: lipid A, a variable polysaccharide chain, and a core sugar. It has been reported that lipid and polysaccharide films can effectively enhance the direct electron transfer between heme proteins and electrodes [5, 6]. We thus expect that an LPS which contains both lipid and polysaccharide chain may provide a new matrix for immobilization of proteins and may be used as a suitable microenvironment for heme proteins to exchange electrons directly with underlying electrodes.

In this study, three heme proteins including hemoglobin (Hb), myoglobin (Mb), and horseradish peroxidase (HRP) were incorporated in LPS films, and the films were modified on pyrolytic graphite (PG) electrodes. Enhanced reversible electron transfer between the heme proteins and underlying PG electrodes was realized. The protein–LPS films have been characterized by UV–Vis absorption spectra, which show that the proteins are not denatured. Furthermore, electrocatalytic reductions of hydrogen peroxide (H$_2$O$_2$) and trichloroacetic acid (TCA) have been observed, showing the potential applicability of the film-modified electrodes as biosensors.

EXPERIMENTAL

Chemicals. Human hemoglobin (MW 66,000), horse heart myoglobin (MW 17,800), horseradish peroxidase (MW 42,000), and lipopolysaccharide were obtained from Sigma. They were all used without further purification. Other chemicals were of analytical grade. All solutions were prepared with double-distilled water, which was purified with a Milli-Q purification system (Branstead, United States) to a specific resistance above 16 MΩ/cm. The solutions were stored in the refrigerator at a temperature of 4℃ when not in use.

Preparation of protein–LPS films. The PG electrode was first polished using rough and fine sand papers. Then its surface was polished to mirror smoothness with an alumina (particle size of about 0.05 μm)/water slurry on silk. Finally, the electrode...
buffers, as is shown in Fig. 1. The anodic and cathodic peaks are located at –0.26 and –0.35 V for Hb, –0.24 and –0.31 V for Mb, and –0.21 and –0.27 V for HRP, respectively. The peak potentials are characteristic for the heme Fe\textsuperscript{III}/Fe\textsuperscript{II} redox couples of the proteins [3, 17, and 18]. Comparably, the PG electrode coated with LPS films alone shows no CV peak in the same potential range (Fig. 1a). Since the bare PG electrode cannot exhibit a voltammetric response effect, the redox peaks in Fig. 1 can be attributed to the redox reactions of the proteins entrapped in the films.

The pairs of the redox peaks of protein–LPS films have an approximately symmetric peak shape and nearly equal heights of reduction and oxidation peaks. The peak currents increase linearly with increasing scan rates from 0.02 to 1 V/s. Integrations of reduction peaks at different scan rates give nearly constant charge (Q) values. All these results are characteristic of quasi-reversible diffusionless thin-layer electrochemistry [19].

The stability of the protein–LPS films modified electrodes was examined. PG electrodes coated with the films are stored in pH 6.0 phosphate buffer, and cyclic voltammograms (CVs) are recorded periodically. All the Hb–, Mb–, and HRP–LPS films demonstrate an excellent stability. The CV peak potentials are at the same positions and the peak currents are nearly stable for at least 2 months.

CVs of the protein–LPS films show a strong dependence on pH. Both the cathodic and anodic peaks potentials of the Fe\textsuperscript{III}/Fe\textsuperscript{II} redox couple shift negatively with an increase in pH. The formal potentials (E\text{\textsubscript{0}}), estimated as the midpoint of cathodic and anodic peaks potentials, have a linear relationship with pH in the range 3.0–10.0. The linear regression equations are $y = -6.8 - 49.7x$ ($R = 0.996$) for Hb–LPS films, $y = 31 - 51x$ ($R = 0.997$) for Mb–LPS films, and $y = 85 - 54.2x$ ($R = 0.997$) for HRP–LPS films. All these slope values are close to the theoretical value of –57.6 mV/pH at 18°C for a single-proton coupled reversible one-electron transfer [20, 21].

In order to check whether the proteins have been denatured after being entrapped in LPS films, UV–Vis spectra of the films were recorded. As is well known, the positions of the sensitive Soret absorption band of the heme prosthetic group for heme proteins can provide information about possible denaturation of the proteins [22, 23]. Taking Hb–LPS as an example, the Soret band of Hb is located at 405.20 nm, which is sensitive to variations of the microenvironments around the heme site. Previous studies have shown that the band should diminish or shift if the protein is denatured [23, 24]. Our experimental results reveal that the Soret band for entrapped Hb is also at 405.20 nm (Fig. 2, the dotted curve), suggesting that Hb in LPS films has a secondary structure similar to the natural state. Mb– and HRP–LPS films demonstrate behaviors very similar to Hb–LPS films.
Further studies reveal that the proteins embedded in LPS films can also exhibit enzymatic activity towards hydrogen peroxide ($H_2O_2$). The electrocatalytic reduction of $H_2O_2$ by Hb in LPS films is shown in Fig. 3. When $H_2O_2$ is added to a pH 6.0 phosphate buffer, an increase in the reduction peak for Hb $Fe^{III}$ at about $-0.35 \text{ V}$ is observed, accompanied by the disappearance of the oxidation peak for Hb $Fe^{II}$. However, no reduction peak of $H_2O_2$ can be obtained at LPS-only modified electrode in this potential range.

The catalytic reduction of $H_2O_2$ at the protein–LPS film electrodes can be used to determine $H_2O_2$ quantitatively. Taking Hb–LPS as an example, the linear relationship between the electrocatalytic reduction peak current and $H_2O_2$ concentration was observed from $1.0 \times 10^{-7}$ to $8.0 \times 10^{-4} \text{ M}$ (Fig. 4). The linear regression equation is $y = 8.152 + 0.04233x$, with a correlation coefficient of 0.999. The detection limit is $4.0 \times 10^{-8} \text{ M}$ with a sensitivity of $0.04233 \mu A/\mu M H_2O_2$. Five independent determinations at a $H_2O_2$ concentration of $50 \mu M$ show a relative standard deviation (R.S.D.) of 3.6%, which displays the nice reproducibility of these measurements. Mb–LPS and HRP–LPS films show similar catalytic behaviors toward $H_2O_2$. The linear ranges for Mb–LPS and HRP–LPS films are $6.0 \times 10^{-7}$–$4.0 \times 10^{-4} \text{ M}$ and $4.0 \times 10^{-7}$–$8.0 \times 10^{-4} \text{ M}$, respectively.

In order to evaluate the catalytic activity of the properties entrapped in LPS film, we calculated the apparent Michaelis-Menten constant ($K_{m}^{app}$) by the Lineweaver-Burk equation [25],

$$I/I_{as} = I/I_{max} + K_{m}^{app}/(I_{max}c),$$

where $I_{as}$ is the steady current after the addition of substrate (with the current derived from heme proteins subtracted), $c$ is the bulk concentration of the substrate, and $I_{max}$ is the maximum current measured under the saturated substrate condition.

$K_{m}^{app}$, which gives an indication of the enzyme–substrate kinetics, is thus calculated to be 1309 $\mu M$ for Hb–LPS films, 1512 $\mu M$ for Mb–LPS films, and 1201 $\mu M$ for HRP–LPS films.

The catalytic activity of the proteins in LPS films toward TCA was also investigated. As shown in Fig. 5, after TCA is added to a pH 6.0 phosphate buffer, the Mb
Fe\textsuperscript{III} reduction peak of Mb–LPS films at about –0.31 V increases. The reduction peak current for Mb Fe\textsuperscript{III} increases with increasing concentration of TCA in solution. However, no catalytic reduction peak of TCA can be observed at the LPS-only modified electrode in the same potential range, which is characteristic of electrochemical catalysis [26, 27]. For Hb–LPS and HRP–LPS films, similar catalytic behaviors toward TCA can be observed.

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