Studies on the direct electrochemistry of hemoglobin immobilized by yeast cells

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The direct electrochemistry of hemoglobin can be achieved by immobilizing hemoglobin onto the surface of yeast cells through electrostatic attractions on a glassy carbon electrode.

Nowadays, studies of the direct electrochemistry of redox proteins at the electrode/solution interface are attracting the interest of more and more scientists. Those researches may provide a kinetic model and a mechanism of electron transfer between biomolecules and enzymes in biological systems, and establish a foundation for fabricating new kinds of biosensors or enzymatic bioreactors. Hemoglobin (Hb) is a heme protein that comprises four polypeptide subunits (two α and two β subunits) with a molar mass of approximately 67000 g mol^{-1}. Although Hb does not function biologically or physiologically as an electron carrier, it can be used as an ideal model molecule for investigation of electron transfer of heme enzymes due to its commercial availability and the known structure. The electron-transfer reactivity of Hb is physiologically hampered and great efforts have been made to enhance it without using mediators and promoters. A protein-film technique[1] has been developed to incorporate Hb into films, such as DNA film, surfactant films, polymer films, protein–polyion layer-by-layer assembly films and natural lipid films. Nanotechnology is also providing a novel way to enhance the electron-transfer rates between Hb and the electrode. Carbon nanotubes,[2] Au colloid[3] and quantum dots[4] are generally used.

Yeast, a typical fungus, has fundamentally the same subcellular structure as higher animal and plant cells. It is usually used as a model system for various basic and applied fields of life science, medicine and biotechnology.[5] *Saccharomyces cerevisiae*, which is the commonly employed yeast cell, has a rigid thick cell wall of about 200 nm in thickness outside of the plasma membrane. The cell wall of *Saccharomyces cerevisiae* is mainly composed of mannoproteins and β-linked glucans, and has a bilayered structure consisting of an internal skeletal layer of glucan, composed of β-1,3- and β-1,6-linked glucose, and a fibrillar or brush-like outer layer composed predominantly of mannoproteins.[6] The application of genetic engineering, using the yeast cell surface as a carrier for protein immobilization, can overcome the limitations of the covalent coupling approach, which is prone to cause changes in the structures of the immobilized proteins or in their characteristics.[7] Imitating genetic engineering, in this work we also immobilized Hb (electropositive) onto the electronegative surface of yeast cells, through electrostatic attractions. On a glassy carbon (GC) electrode, the direct electrochemistry of Hb was achieved, similar to other protein films. Hb immobilized in yeast cell film retained its biological activity and gave sensitive electrochemical reduction signals involving reactions with NO and H_2O_2.

The Hb/yeast/GC electrode was prepared by the following procedure. The bare GC electrode was first polished with 0.05 μm alumina slurry, and then sonicated in nitric acid (1 : 1), ethanol and doubly-distilled water in turn. Yeast cells were precultured in 5 mL YPD (1.0% yeast extract, 2.0% tryptone, 2.0% glucose) with shaking at 30 °C overnight. A 2.0% dilution of the cells was grown in 5 mL YPD with shaking at 30 °C for 10 h, harvested by centrifugation at 2500 rpm for 3 min and washed three times with sterile water. Then the pellets were resuspended in sterile water at a concentration of 10^8 cells/mL. 5 μL of the resuspended solution was cast onto the GC electrode and dried at ambient temperature. Afterwards, the yeast cell modified GC electrode was immersed in the Hb solution (3 mg/mL, dissolved in 0.1 mol/L pH 5.0 phosphate buffer solution, PBS) for 3 h. The final electrode was denoted as Hb/yeast/GC electrode.

Fig. 1 shows cyclic voltammograms (CV) of three electrodes at 0.2 V s^{-1} in pH 7.3 buffers. There are no redox peaks at the bare GC electrode (curve b) and the yeast/GC electrode (curve c). Meanwhile, a pair of well-defined and quasi-reversible redox peaks (curve a) with a formal potential ($E^{\circ\prime}$) of −0.349 V (*vs.* saturated calomel electrode, SCE) is observed at Hb/yeast/GC electrode, reflecting the direct electrochemistry of Hb. The anodic and cathodic peak potentials are located at −0.321 and −0.377 V, respectively. The ratio of the cathodic current over the anodic one is close to 1, and the reduction and oxidation peak currents (i_p) exhibited a linear relationship with the scan rate in the range of

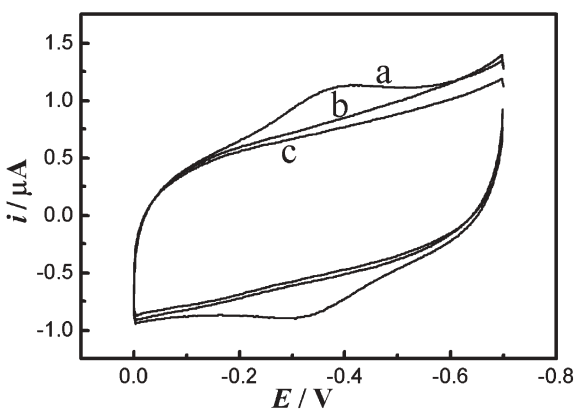

Fig. 1 Cyclic voltammograms at 0.2 V s^{-1} in pH 7.3 buffers of Hb/yeast/GC electrode (a), bare GC electrode (b) and yeast/GC electrode (c).

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0.06–1.0 V s^{-1}. Peak width at half-height is well in excess of the theoretical 90 mV for a one-electron surface reaction, and peak separation (56 mV) is in excess of the ideal thin-film value of 0 mV.[8] The charge consumed in coulombs, Q, obtained from integrating the anodic or cathodic peak area in cyclic voltammograms under the background correction was invariable in substance. Furthermore, the log i_{pc}–log v and log i_{pa}–log v plots were linear with the ratio of the slopes about one. These CV characteristics are fairly typical for metalloprotein–lipid films and reflect quasireversible nonideal thin protein film voltammetry, and the electrochemical reaction of Hb on the yeast/GC electrode is a surface-controlled behavior, not a diffusion-controlled one.[1] The surface concentration of electroactive Hb (Γ^*) could be estimated from integration of the reduction peak in the CVs according to the following equation:

$$Q = nFA\Gamma^*$$

The value of Γ^* for Hb at the yeast/GC electrode was calculated with an average value of 5.55×10^{-12} mol/cm^2. However, the total amount of Hb modified on the electrode was estimated to be about 1.32×10^{-11} mol/cm^2, referring to the absorbance data obtained by ultraviolet-visible (UV-Vis) spectroscopy. Comparing the values above, there was about 42.0% of the total amount of Hb immobilized on the electrode involved in the electron-transfer reaction.

Nearly reversible voltammograms with well-defined redox peaks were obtained for Hb at the yeast/GC electrode in the pH range from 4.0 to 9.0. The pH increase caused a negative shift in $E^{\circ\prime}$. As is known, decreasing the solution pH will increase $E^{\circ\prime}$ of many heme-proteins that undergo proton-coupled electron transfer. This redox Bohr effect, a shift in $E^{\circ\prime}$ as a function of pH, results from the change in the protonation of a water molecule at the sixth coordination position in the heme iron and protonation of the protolytic groups around the heme with changing pH.[9] Thus, the linear relationship between the shift in $E^{\circ\prime}$ of the Hb/yeast/GC electrode and the solution pH suggested that the redox reaction was accompanied by the transfer of protons. Theoretically, the slope of $E^{\circ\prime}$ *vs.* pH for a single-proton coupled reversible one-electron transfer is −0.059 V/pH. However, it is −0.033 V/pH in this work, much smaller than the theoretical value. The reason for this relates to the influence of the protonation of *trans* and residue ligands around the heme and the protonation of the water molecules coordinated with the central iron.[10]

It is believed that Hb could be immobilized onto the surface of yeast cells through electrostatic attractions, which were thoroughly studied and validated in the following experiments. Firstly, UV-Vis spectroscopy and fluorescence microscopy (FM) were performed to examine the electrostatic attractions between them when Hb was mixed with yeast cells in solution. The Hb solution (the same concentration as above) was mixed with the resuspended yeast cell solution (prepared as above) at 30 °C with shaking for 24 h. Then the mixture was harvested by centrifugation at 2500 rpm for 3 min to obtain the upper clear liquid, which was used as the Hb/yeast sample in UV-Vis spectroscopy. Following the Beer–Lambert law, the intensity of the Soret absorption band of the Hb/yeast sample became more decrescent than that of the simplex Hb solution, suggesting that some Hb molecules had been adsorbed onto the surface of yeast cells.

During the process of FM, Hb was combined with water-soluble quantum dots through a covalent bond, which was used as a fluorescent probe. For each experiment, the pellets of the yeast cells (prepared as above) resuspended in 1.5 mL sterile water were incubated with 20 μL Hb–QDs conjugates with shaking at 30 °C for 24 h. The cell suspensions were then harvested by centrifugation at 2000 rpm for 3 min, washed three times with sterile water to remove free Hb–QDs conjugates, and then resuspended in sterile water. Fig. 2 shows the fluorescence images of yeast cells (left) and yeast cells conjugated with Hb–QDs (right). A binding site of Hb–QDs on a yeast cell surface can be found easily (right), indicating Hb molecules have been adsorbed onto the surface of yeast cells. In the control experiment, when Hb was dissolved in pH 7.0 PBS, there is no binding site observed (not shown). Yeast cells and QDs have negative charges, while Hb in pH 5.0 PBS has positive charges. Only the electrostatic attractions existing between Hb and yeast cells can lead to these phenomena.

In order to simulate the situation of the yeast cells cast onto the GC electrode, FM and environmental scanning electron microscopy (ESEM) were carried out to study the dry films of yeast cells and Hb/yeast. Fig. 3 shows the fluorescence image of a dry film of yeast cells subjected to immersion in Hb solution for 3 h. Comparing the bright field image (left) and fluorescent image (right), it can be obviously concluded that Hb–QDs conjugates overlay the surface of yeast cells in a large area. Yeast cells would adsorb more Hb molecules when dried than when they were in solution. From ESEM (Fig. 4), the morphology of the dry film of yeast cells showed that the single cells scattered one by one (left) under the same conditions. However, when the dry film of yeast cells was immersed in Hb solution, the morphology illustrated an alveolate structure (right). Considering the phenomenon of FM above, the alveolate structure may be the Hb molecules that were adsorbed onto the surface of yeast cells layer by layer.

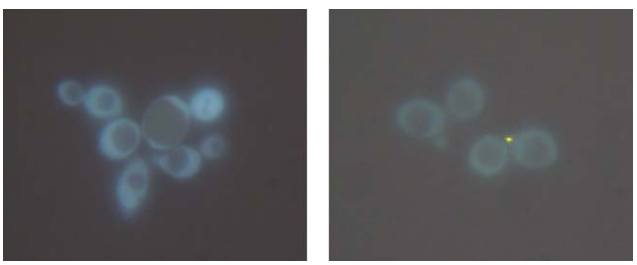

Fig. 2 Fluorescent images of yeast cells in sterile water only (left) and yeast cells conjugated with Hb–QDs in sterile water (right) using an inverted fluorescence microscope.

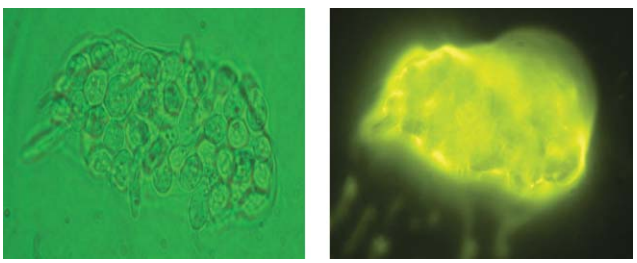

Fig. 3 Fluorescence microscopy imaging of the surface of yeast cells' film after being immersed in Hb solution: bright field image (left) and fluorescent image (right).

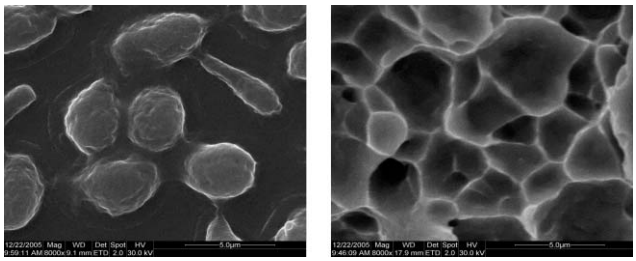

Fig. 4 ESEM views with a magnification of 8000: dry film of yeast cells (left) and dry film of yeast cells immersed in Hb solution (right).

The influence of the ionic strength on the electrostatic attractions and the electrochemical response was also examined. The precultured yeast cells were resuspended in 20 mL ultrafiltered distilled water containing a given quantity of NaCl with respect to the next CV experiments to be performed. Four concentrations of saline solution were tested, corresponding to the ionic strength of 1, 5, 20 and 100 mM, respectively. The increase of ionic strength led to the slight decrease of i_p, which resulted in lower electrostatic attractions between Hb molecules and yeast cells. The value of i_p lies on the surface concentration of electroactive Hb at yeast/GC electrode. Although the increase of ionic strength reduced the total amount of Hb adsorbed, its influence on i_p was not pronounced.

The position of the sensitive Soret absorption band of the heme prosthetic group for heme proteins provides additional information about the possible denaturation of proteins, especially about conformational change in the heme group region. When cast onto crystal glass slides, the dry film of yeast cells didn't show the position of the Soret absorption band, however the Hb/yeast film gave almost the same peak position of the Soret band as that of dry Hb film alone. The bioelectrocatalytic activity of Hb immobilized in yeast cell film was also confirmed by experiments that catalyzed the reduction of NO and H_2O_2. The reduction peak potential of NO located at −0.846 V exhibited a linear relationship with the NO concentration in the range of 1.28 μM to 14.4 μM (R = 0.995, n = 15). The same result was obtained for H_2O_2, within the range of 1.08 μM to 79.2 μM (R = 0.999, n = 18); the catalytic reduction peak current was linearly related to the H_2O_2 concentration. In control experiments, the yeast/GC electrode had no electrocatalytic activity.

In summary, Hb can be immobilized by yeast cells on the surface of the GC electrode, and the direct electrochemistry of Hb was achieved. UV-Vis, FM and ESEM were used to validate the interaction of Hb and yeast cells. The bioactivity of Hb immobilized in yeast cell film is retained, and the catalytic reduction of NO and H_2O_2 was estimated.

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