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Short communication

Direct electron transfer and electrocatalysis of myoglobin loaded in layer-by-layer films assembled with nonionic poly(ethylene glycol) and ZrO_2 nanoparticles

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ABSTRACT

Nonionic poly(ethylene glycol) (PEG) and ZrO_2 nanoparticles were successfully assembled into $\{PEG/ZrO_2\}_n$ layer-by-layer films on solid surfaces through coordination interaction between the ether oxygen groups in PEG and the Zr(IV) in ZrO_2 nanoparticles. The $\{PEG/ZrO_2\}_n$ films were then immersed in myoglobin (Mb) solution at pH 5.0 to load Mb into the films, designated as $\{PEG/ZrO_2\}_n$–Mb. Cyclic voltammetry (CV), quartz crystal microbalance (QCM), and scanning electron microscopy (SEM) were used to characterize both $\{PEG/ZrO_2\}_n$ and $\{PEG/ZrO_2\}_n$–Mb films. Mb in the $\{PEG/ZrO_2\}_n$–Mb films fabricated on pyrolytic graphite (PG) electrodes showed direct and quasi-reversible CV response, which could be used to electrocatalyze reduction of oxygen and hydrogen peroxide. The interaction between Mb and $\{PEG/ZrO_2\}_n$ films in loading was also discussed and explored. The results suggest that the electrostatic interaction is the main driving force for the loading of Mb into the $\{PEG/ZrO_2\}_n$ films, while hydrogen bonding and/or hydrophobic interaction are also important factors for stabilizing $\{PEG/ZrO_2\}_n$–Mb films in blank buffers. The comparative experiments demonstrated that only those heme proteins whose dimension was smaller than the average pore size of the films were able to be loaded into the films and exhibited electroactivity.

1. Introduction

The immobilization of enzymes on solid substrates without changing the native structure and bioactivity of the enzymes is a challenging task in biotechnology, biodevices, and other bio-related area, and arouses increasing interest. Among various immobilization approaches, layer-by-layer assembly demonstrates obvious advantages for its precise control of film thickness, extremely simple procedure, and the predesigned architecture at molecular level [1]. This approach has now been extended to constructing enzyme or protein films with oppositely charged polyions or nanoparticles [2,3], and the direct electrochemistry of some redox proteins in these multilayer films on electrodes without using any mediator has also been realized [4–8]. The study of direct electrochemistry of redox proteins may provide a model for the mechanism study of electron transfer between enzymes in real biological systems, and can establish a foundation for fabricating the third-generation biosensors without using mediators [9,10].

Recently, a novel technique for fabricating protein films has been proposed based on the layer-by-layer assembly. After the layer-by-layer films of polyelectrolytes and/or nanoparticles are assembled on solid substrates, they are immersed into protein solutions to load or "absorb" the proteins into the films, forming protein-loaded layer-by-layer films [11–13]. Compared with the regular protein layer-by-layer films directly assembled by proteins and polyelectrolytes or nanoparticles, the protein-loaded layer-by-layer films demonstrate unique advantages [14–17]. For example, the multilayer films can be fabricated under some "extreme" conditions, such as in organic solvent, with strong acid or base, or at high temperature, without affecting the original conformation and bioactivity of loaded proteins because the immobilization of proteins is independent of the assembly process and the loading of proteins is completely spontaneous.

However, the loading process of polyelectrolyte multilayer films toward proteins is usually extremely slow, and the immobilization amount of proteins is often very limited, mainly because of the poor permeability or porosity of most layer-by-layer films and the relatively large size of proteins. Only those multilayer films with appropriate components, suitable structure and good porosity can demonstrate good permeability toward some specific proteins and effectively load the proteins [17]. Nanoparticles, with their rigid structure and good biocompatibility, should thus be a good candidate in this respect. The multilayer films containing rigid nanoparticles might demonstrate better porosity than those assembled with "soft" polyelectrolytes, and should be more suitable for protein loading [18]. Recently, our group reported the loading behavior of $\{PDDA/SiO_2\}_n$ films toward myoglobin (Mb) [19]. Compared with $\{PDDA/PSS\}_n$ films, the $\{PDDA/SiO_2\}_n$ films assembled with PDDA and SiO_2 nanoparticles showed better permeability and more loading amounts of Mb. The direct electrochemistry of redox proteins has also been realized in $\{PDDA/SiO_2\}_n$–Mb films and other protein-loaded layer-by-layer films [14–17].

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In the present work, poly(ethylene glycol) (PEG) and ZrO_2 nanoparticles were assembled layer-by-layer into $\{PEG/ZrO_2\}_n$ films on the surface of pyrolytic graphite (PG) electrodes, and the films were then immersed in Mb solution to load Mb, forming $\{PEG/ZrO_2\}_n$–Mb films. The direct electron transfer of Mb in the films with underlying PG electrodes was realized and used to catalyze different substrates. Various techniques such as cyclic voltammetry (CV), quartz crystal microbalance (QCM), and scanning electron microscopy (SEM) were used to characterize the $\{PEG/ZrO_2\}_n$ and $\{PEG/ZrO_2\}_n$–Mb films. Here, PEG was selected as one of the building blocks of $\{PEG/ZrO_2\}_n$ films mainly because PEG could provide a favorable microenvironment for heme proteins to realize their direct electrochemistry in their cast or layer-by-layer films with PEG [20–22]. ZrO_2 nanoparticles were chosen mainly because of their good biocompatibility in retaining the native structure and bioactivity of biomacromolecules. For instance, the heme proteins immobilized in ZrO_2 nanoparticle films exhibited direct and reversible CV responses for their heme Fe(III)/Fe(II) redox couples and were used to electrocatalyze reduction of hydrogen peroxide [14,23,24]. However, to the best of our knowledge, the layer-by-layer assembly of PEG and ZrO_2 nanoparticles, the loading behavior of the $\{PEG/ZrO_2\}_n$ films toward heme proteins, and the direct electrochemistry and electrocatalysis of the $\{PEG/ZrO_2\}_n$–Mb films have not been reported up to now.

The driving force of layer-by-layer assembly is usually thought to be electrostatic interaction between oppositely charged components [1]. However, PEG is a neutral nonionic polymer with no charge on its backbone, while ZrO_2 nanoparticle carries negative charges in its aqueous suspension [25]. Therefore, the driving force of the assembly of $\{PEG/ZrO_2\}_n$ multilayer films could not be attributed to electrostatic interaction. In this work, the assembly driving force of the $\{PEG/ZrO_2\}_n$ films and the possible mechanism of Mb incorporation into the films were also explored and discussed. A better understanding of the interactions involved in the process of film assembly and protein loading will guide us to find novel and more suitable protein-loaded film systems in the future.

2. Experimental section

2.1. Chemicals

Horse heart myoglobin (Mb, MW 17,800), bovine hemoglobin (Hb, MW 66,000), bovine liver catalase (Cat, 15,000 units mg^{-1}, MW 240,000), poly(ethylene glycol) (PEG, MW 2000), 3-mercapto-1-propanesulfonate (MPS, 90%), poly(diallyldimethylammonium chloride) (PDDA, 20%, MW 200,000–350,000), and zirconium dioxide nanoparticles (diameter 20–30 nm) were purchased from Sigma-Aldrich and used as received without further purification. Hydrogen peroxide (H_2O_2, 30%) were obtained from Beijing Chemical Plant and freshly prepared before being used. All other chemicals were of reagent grade, and water was purified twice by ion exchange and subsequent distillation.

2.2. Film assembly

For electrochemical experiments, the layer-by-layer films were assembled on basal plane pyrolytic graphite (PG, Advanced Ceramics, geometric area 0.16 cm^2) disk electrodes. Prior to assembly, the PG electrodes were polished on 400-grit metallographic sandpaper, ultrasonicated in water for 20 s, and then dried in air. The electrodes were immersed into PDDA solutions (3 mg mL^{-1}, containing 0.5 M NaCl) for 20 min to adsorb a PDDA precursor layer, making the electrode surface become positively charged and smoother. The PG/PDDA electrodes were then alternately placed into PEG solutions (1 mg mL^{-1}, containing 0.1 M NaCl at pH 5.4) and ZrO_2 aqueous suspensions (1 mg mL^{-1}) for 20 min with intermediate water washing and air drying. The adsorption cycle was repeated to obtain the $\{PEG/ZrO_2\}_n$ layer-by-layer films on the PG/PDDA surface, where n represents the number of bilayers. For loading of Mb, the multilayer film electrodes were immersed in Mb solutions (1 mg mL^{-1}, containing 0.3 M NaCl at pH 5.0) for a certain time, and the Mb-loaded films were designated as $\{PEG/ZrO_2\}_n$–Mb. The $\{PEG/ZrO_2\}_n$–Mb films were then transferred in pH 7.0 buffers containing no Mb for CV scans. Other proteins or enzymes, such as Hb (1 mg mL^{-1} at pH 5.0) and Cat (10 mg mL^{-1} at pH 5.0), were loaded into the $\{PEG/ZrO_2\}_n$ films in the same way.

For QCM studies, the gold electrodes (geometric area 0.196 cm^2) coated on both sides of quartz crystal resonator disk (fundamental frequency 8 MHz) were pretreated with freshly prepared piranha solution (3:7 volume ratio of 30% H_2O_2 and 98% H_2SO_4. Caution: piranha solution should be handled with extreme care, and only small volumes should be prepared at any time). After being washed with ethanol and water thoroughly, the QCM gold electrodes were immersed in 4 mM MPS ethanol solutions for 24 h to chemisorb an MPS monolayer and make the surface become negatively charged. The PDDA precursor layer and following $\{PEG/ZrO_2\}_n$ films were then assembled on the Au/MPS surface with the same procedure as that on the PG electrodes. After each adsorption step, the QCM gold resonator electrodes were washed in water, dried in a nitrogen stream, and then measured by QCM in air.

2.3. Apparatus and procedures

A CHI 660A electrochemical workstation (CH Instruments) was used for all electrochemical measurements. A regular three-electrode cell was used with a saturated calomel electrode (SCE) as the reference, a platinum wire as the counter, and a PG disk electrode coated with films as the working electrode. Before electrochemical measurements, buffer solutions were purged with purified nitrogen for at least 15 min. A nitrogen atmosphere was then kept in the cell by continuously bubbling N_2 during the whole experiment.

QCM measurements were performed with a CHI 420 electrochemical analyzer. According to the Sauerbrey equation [26], the following relationship is obtained between adsorption mass, ΔM (g), and frequency shift, Δf (Hz), by taking into account the properties of quartz resonator used in this work: $\Delta f = -7.40 \times 10^8 \Delta M$. Thus, 1 Hz of frequency decrease corresponds to 1.35 ng of mass increase.

Scanning electron microscopy (SEM) was run with an S-4800 scanning electron microanalyzer (Hitachi) at an acceleration voltage of 5 kV. The PEG/ZrO_2 multilayer films assembled on PG/PDDA surface were used as samples. Prior to SEM analysis, the films were coated with about 10 nm of Au by an IB-3 ion coater (Eiko).

All experiments were done at ambient temperature (20±2 °C).

3. Results and discussion

3.1. Assembly of $\{PEG/ZrO_2\}_n$ layer-by-layer films

QCM was used to monitor or confirm the assembly of $\{PEG/ZrO_2\}_n$ layer-by-layer films (Fig. 1). The results showed a non-linear decrease of frequency with the adsorption step, indicating that the $\{PEG/ZrO_2\}_n$ films could be successfully assembled on Au/MPS/PDDA surface.

PEG is a type of neutral polymer with no charge on its backbone, while ZrO_2 nanoparticle carries negative charges in its aqueous suspension with the isoelectric point at 4.15 [25]. Therefore, the driving force of the assembly of $\{PEG/ZrO_2\}_n$ multilayer films could not be ascribed to electrostatic interaction between PEG and ZrO_2. One PEG molecule contains a great number of ether oxygen groups in its ethylene oxide structure. This kind of oxygen has one active unshared lone pair of electrons, and thus has a high coordination capability as the electron donor [27,28]. Zr(IV), on the other hand, has many unoccupied orbitals and can accept at most 8 lone pairs of electrons, and thus shows good coordination capability as the electron acceptor.