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Nanoporous gold film encapsulating cytochrome *c* for the fabrication of a H_2O_2 biosensor

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ABSTRACT

A layer-by-layer route to prepare nanoporous Au film materials on transparent ITO substrates is reported by alternatively assembling Au and Ag nanoparticles through 1,5-pentanedithiol as a cross-linker, followed by that Ag nanoparticles are dissolved at room temperature in $HAuCl_4$ solution. Electron transfer of cytochrome *c* (cyt. *c*) – an excellent model for investigation of biomolecules, is greatly facilitated at the nanoporous Au film with electron transfer rate constant (k_s) of 3.9 s^{-1}. Meanwhile, cyt. *c* adsorped onto the nanoporous Au film still maintain its enzymatic activity toward H_2O_2. On the basis of these experimental results, the cyt. *c*-nanoporous Au film is exploited to an amperometric biosensor for H_2O_2 with high selectivity, broad linear range, low detection limit, and long stability.

1. Introduction

Porous Au has attracted significant attention recently for various applications including catalysis, fuel cells [1,2], cell imaging [3], energy storage [4], investigation of the critical behavior of liquid helium [5] and development of chemical sensors [6] due to its high surface-to-volume ratio, stability, and biocompatibility. The preparation of porous Au has been reported by the formation of a gold/silver alloy used as a precursor, followed by dealloying with concentrated nitric acid during which silver is removed [7]. Other methods, such as thermal decomposition of Au_2O_3, sublimation of iodine from Au–I_2 pressed powders [8], and dissolution of gold chloride in a dextran solution followed by heating to 600–800 °C [9] have been developed. Porous Au has also been prepared by means of electrochemical deposition from the homogeneous hexagonal mesophase of nonionic surfactants with metal salts [10]. The layer-by-layer (LbL) assembly technique has the advantage of providing a simple and versatile way to assemble metallic colloids into ultrathin films in which the film structure, thickness, and composition can be well tailored. If a multilayer film of colloidal Au alternately assembled with a pore-forming species is prepared, a gold film with interconnected and controlled pores could be prepared after the removal of the pore-forming species from the film. Taking advantage of this concept, we prepared nanoporous Au film on the transparent ITO by selective dissolution of sacrificial templates of silver particles in colloidal Au/Ag multilayer.

One of the most attractive avenues for the application of porous material is its use as a solid support for the immobilization of biomolecules such as proteins and enzymes. The successful immobilization of biomolecules onto solid substrates is a crucial, fundamental event for many aspects of bioanalytical chemistry, including biosensor engineering [11], biological electron transfer modeling systems [12] and the development of biocompatible materials [13]. Significant research efforts have focused on understanding and controlling protein adsorption at man-made substrates either as a precursor to a biosensor design or as a means of exploring fundamental properties of biological systems. A prominent example of this type of research is the direct or unmediated electrochemistry of redox-active proteins at modified electrodes [14–16]. Cyt. *c* is an excellent model for studying the electron transfer of typical metalloproteins from a structure point of view. Up to now, a number of methods have been developed to realize the direct electron transfer of cyt. *c* and subsequently to construct the H_2O_2 biosensor. Au nanoparticles (AuNPs), due to its prominent properties such as large surface-to-volume ratio, good biocompatibility, high catalytic efficiency, and strong adsorption ability, have been showing great applications in the construction of modified electrodes for protein immobilization. Several modified electrodes prepared with AuNPs have been fabricated to encapsulate proteins which have also displayed good direct electron transfer and catalytic activity [17–20].

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In this work, AuNPs were firstly employed, together with LBL and SAM techniques, to construct a nanoporous Au film on transparent ITO surface using colloidal silver as a pore-forming species in the colloidal Au/Ag multilayer films, for the immobilization of cyt. *c*. The purely porous Au nanostructures were fabricated in a mild and template-independent way, together with the potential ability to provide a uniform surface environment for the adsorption of biomolecules across a large surface area.

2. Experimental

2.1. Materials

Hydrogen tetrachloroaurate ($HAuCl_4 \cdot 3H_2O$), 1,5-pentanedithiol (noted as dithiol), poly(diallyldimethylammonium chloride) (PDDA, aqueous solution with a molecular weight of 100,000–200,000), horse heart cytochrome *c* (cyt. *c*, MW 12,384) were purchased from Sigma–Aldrich. All other reagents were of analytical grade, and double distilled water was used throughout. ITO-coated glass plates with a square resistance of $\sim 10\,\Omega\,cm^{-2}$ were purchased from Shenzhen Nanbo Display Technology Co. Ltd. (Shenzhen, China).

2.2. Preparation and modification of nanoporous Au film

The Au colloids were prepared by the conventional $NaBH_4$ reduction of $HAuCl_4$ in water [21]. The colloidal Ag nanoparticles were prepared in a similar way to that of colloidal Au except $HAuCl_4$ was replaced with $AgNO_3$. Transmission electron microscopy (TEM) revealed an average diameter of 5 ± 1.2 nm and 10 ± 2.4 nm for colloidal Au and Ag respectively (Supplementary Information, Fig. S1). ITO-coated glass plate was used as substrate and was cleaned by sonicating sequentially for about 30 min each in acetone, 10% KOH in ethanol, and distilled water.

As schematically shown in Fig. 1, the prepared ITO electrode with original negative surface charge was first treated with an aqueous solution of PDDA (1 mg/mL) for about 30 min to form positively charged surface. After the treated substrate was washed with distilled water and dried, it was immersed in colloidal Au solution for 5 min followed by extensively rinsing with water and drying with nitrogen gas. This substrate was then immersed in a 10 mM alcoholic solution of 1,5-pentanedithiol for 5 min followed by rinsing with abundant ethanol and drying with nitrogen gas. The 1,5-pentanedithiol-covered substrate was then adsorped in Ag colloids for 5 min followed by extensively rinsing with water and drying with nitrogen gas to obtain a layer of Ag colloids. By repeating the above steps in a cyclic fashion, composite multilayer films $(Au/Ag)^*n$ with *n* referring to the number of deposition cycles was fabricated. After complete dissolution of Ag nanoparticles by immersing the multilayer films in a mixture of an aqueous solution of 3.0 mM $HAuCl_4$ and 3 M NaCl, a nanoporous Au film was obtained. The as-prepared nanoporous Au film was adsorped in 25 mM PBS (pH 7.2) containing 0.2 mM cyt. *c* for about 30 min at 4 °C in refrigerator. Hereafter, cyt. *c* modified nanoporous Au electrode will be referred as cyt. *c*/porous Au/ITO.

2.3. Instrumentations and measurements

Cyclic voltammetric (CV) and amperometric measurements were carried out by a computer-controlled CHI 660C electrochemical workstation (Shanghai, China) in a home-made three-electrode electrochemical cell using a KCl-saturated Ag/AgCl electrode as reference electrode and a platinum wire as auxiliary electrode. The supporting electrolyte was 25 mM phosphate buffer solution (PBS, pH 7.2), which was prepared with KH_2PO_4 and K_2HPO_4. The PBS was purged with high-purity nitrogen for at least 30 min prior to experiments and a nitrogen atmosphere was kept over the solution in the cell. In steady-state amperometric experiment, the potential was set as at −0.1 V with a stirring rate of 200 rpm, and the current–time curves were recorded after a constant background current had been established. All electrochemical experiments were performed at the room temperature. UV–vis absorption spectrum was recorded by an Agilent 8453 UV–vis–NIR spectrophotometer (Agilent instruments). SEM images were taken by a Quanta 200 FEG (FEI Company). TEM images were taken by a JEM-1230 (JEOL Company).

3. Results and discussion

3.1. Characterization of nanoporous Au films

To prepare nanoporous Au films, a precursor film in which Ag nanoparticles are well dispersed in layers of Au nanoparticles are required. For this aim, multilayer films comprised of nonsaturated layers of colloidal Au and Ag were employed. UV–vis spectra of colloidal Au and Ag in solution show a plasmon absorption peak at 519 and 395 nm, respectively (Supplementary Information, Fig. S2). These plasmon absorption peaks remain in the solid films with a slight red-shift compared with those in solution and therefore can be used to monitor the adsorption dynamics of colloidal Au and Ag. Colloidal Au or Ag was adsorbed at different time intervals on a 1,5-pentanedithiol-modified ITO slide, and its absorbance at λ_{max} was recorded. The absorbance both increased drastically in the beginning and gradually reached constant after 20 min of adsorption. Herein, a 5 min immersion time in colloidal Au and Ag solutions was used to construct Au/Ag multilayer.

The construction process of the composite Au/Ag multilayer on ITO slides was monitored by UV–vis spectroscopy. As shown in Fig. 2, the first adsorbed layer (line 1) of Au nanoparticles has a plasmon absorption peak at 531 nm, which is 12 nm red-shifted compared with that in solution due to the change of refractive index. When a second layer (line 2) of Ag nanoparticles was adsorbed, the typical plasmon absorption peak of nonaggregated colloidal Ag at 400 nm appeared with the absorbance between 200 and 700 nm all increasing at the same time. The absorbance

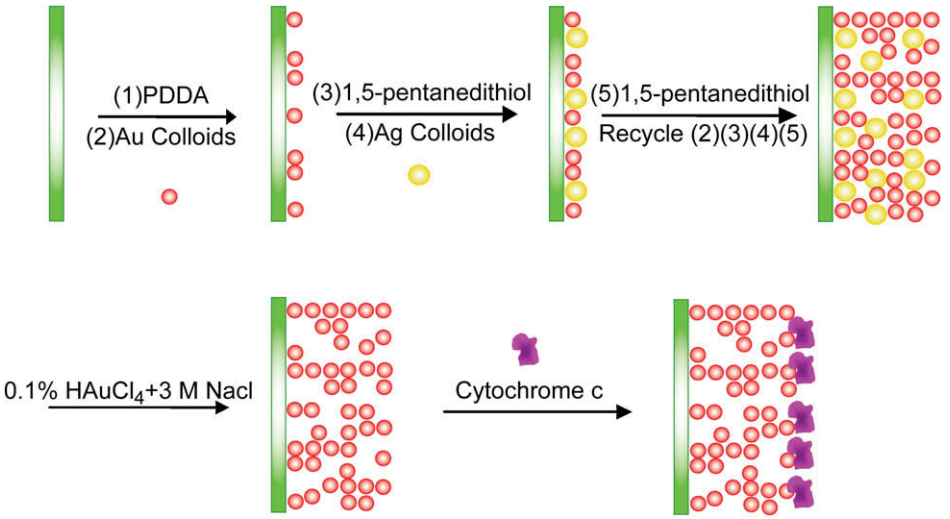

Fig. 1. Schematic illustration of preparing process of cyt. *c*/nanoporous Au/ITO electrode.