Original Research

In-situ secondary growth of nanocube-based Prussian-blue film as an ultrasensitive biosensor☆

Yu Liu, Jingmeng Peng, Danfeng Jiang, Zhenyu Chu, Wanqin Jin*

State Key Laboratory of Materials-Oriented Chemical Engineering, Jiangsu National Synergetic Innovation Center for Advanced Materials, College of Chemical Engineering, Nanjing Tech University, 5 Xinmofan Road, Nanjing 210009, China

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ABSTRACT

A regular nanostructure has been widely confirmed to result in a marked improvement in material performance in biosensing applications. In the present study, a regular nanostructured Prussian blue (PB) film with two heterogeneous crystal layers was synthesized *in-situ* using a secondary growth method. A PB seed layer was first controlled to form uniform cube-like crystal nuclei through an ultrasonic reaction with a single reactant. Then, well-defined 100 nm PB nanocubes were further crystallized on this seed layer using a self-assembly approach. In order to accelerate the electron transfer rate during the enzyme reaction for glucose detection, the graphene was used as the main cross-linker to immobilize glucose oxidase on the PB film. The as-prepared biosensor exhibited high electrocatalysis and electron conductivity for the detection of trace glucose with a sensitivity of $141.5\ \mu A\ mM^{-1}\ cm^{-2}$, as well as excellent anti-interference ability in the presence of ascorbic acid and uric acid under a low operation potential of $-0.05\ V$.

1. Introduction

In recent decades, biosensor research has achieved significant progress and improvement due to continuously emerging sensing materials. The most important biosensor parameters are sensitivity, selectivity and stability [1–3]. Previously, the scientists believed that the above features were mainly determined by the natural properties of adopted electrode materials [4]. Hence, earlier studies mainly focused on the selection and synthesis of new materials for bioanalysis. However, the current nanomaterial research has demonstrated that the formation of regular nanostructures can greatly improve biosensor performance by promoting catalysis and conductivity [5,6]. Therefore, the design of regular material nanostructures for biosensor fabrication is attracting increasing interest.

Prussian blue (PB), which is known as "artificial peroxidase" [7] due to its excellent electrocatalytic ability for H_2O_2, is used for biosensing analysis of various physiological substances with immobilization of matching oxidases [8]. However, traditional synthesis methods, such as chemical deposition and electrodeposition, cannot harvest PB regular nanostructures due to a fast formation rate which causes excess crystal nuclei and rapid crystallization. In order to resolve this deficiency, Vaucher et al. [9] applied a reverse microemulsion method to obtain regular nanostructured PB powders using an anionic surfactant.

Subsequently, Wu et al. [10] developed a sonochemical synthesis route to prepare single-crystalline nanocubic PB crystals with PVP protection. However, these approaches are not able to achieve the *in-situ* formation of regular PB on the electrode surface, which requires an extra immobilization step before application. In our previous studies [11–13], a series of *in-situ* methods were developed to realize the regular growth of PB crystals for performance enhancement. Nevertheless, these structures and the distribution of PB crystals were not integrated and uniform enough, which caused an increase in resistance and a decrease in the catalytic area. Recently, we developed a low-speed chemical synthesis technique to prepare regular PB nano-crystals for a screen-printing ink [14]. Although the geometric structure of the synthesized PB nanocubes was well-defined and uniform, this approach cannot be directly applied to the *in-situ* growth of PB on an electrode. Previous studies [15–17] have shown that the characteristics of a crystal seed determine the crystallization behaviour and the final morphology. PB cannot maintain stability under a high temperature [18], therefore, the widely used hydrothermal synthesis method is not suitable for PB seed formation. In order to obtain a regular and uniform PB film, the seed should be small in size with a uniform distribution. Consequently, the initial stage of PB nucleus formation should be well controlled by the specially designed strategy.

In this study, a uniform PB film with well-defined nanocubic

crystals was successfully prepared using a secondary synthesis method. A seed layer with 50 nm cube-like PB crystals was first formed by ultrasonic deposition. A self-assembly approach was subsequently used to realize further regular growth of isolated PB crystals 100 nm in size by controlling the assembled layers. Following cross-linking of glucose oxidase by the graphene/glutaraldehyde mixture, the as-prepared biosensor exhibited ultra-sensitivity and excellent anti-interference ability in the detection of glucose under avery low potential.

2. Experimental section

2.1. Reagents and apparatus

Analytic grade $K_4[Fe(CN)_6]\cdot 3H_2O$, $FeCl_3\cdot 6H_2O$, glutaraldehyde, ascorbic acid and uric acid were purchased from Sigma-Aldrich. Five milligrams of glucose oxidase (GOD, EC 1.1.3.4, 168800 units/g, from *Aspergillusniger*, Sigma-Aldrich), 30 wt% H_2O_2 (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China), glucose (Sinopharm Chemical Reagent Co., Ltd.) and graphene powder (Jcnano Technology Co., China) were used directly without purification. All solutions were prepared using deionized water.

The electrochemical tests were performed in 0.1 M PBS at 25 °C using an electrochemical workstation (CHI 660C, Shanghai Chenhua, China). Platinum (Pt) wire and Ag/AgCl were used as the counter and reference electrodes, respectively. The scan rate in cyclic voltammetry (CV) characterization was 50 mV/s. Electrochemical impedance spectroscopy (EIS) measurement of the voltage amplitude at 5 mV in the frequency range 0.05–105 Hz was obtained in the 0.01 M $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ solution under an initial potential of 0 V. Online monitoring of PB growth was carried out using a quartz crystal microbalance (QCM200, Stanford Research Systems, Inc., USA). All field emission scanning electron microscopy (FESEM) images were obtained using a Hitachi-4800. An atomic force microscope (AFM) (AFM, XE-100, Park Systems, Korea) was used to obtain information on the morphology of the PB film. The images were collected in non-contact mode.

2.2. Ultrasonic deposition of the PB seed layer

The Au disk electrode (diameter=2 mm) was pretreated with polish and metallographical sandpaper until a mirror-like surface was obtained. The electrode was then washed in deionized water with ultrasonic vibration to remove small residual particles of sandpaper. The electrode was then immersed in the 0.01 M $K_4[Fe(CN)_6]$ solution, and the concentration was changed as necessary. A 25 kHz ultrasonic wave was then used continuously for 1 h. The prepared electrode was then washed with deionized water and dried to complete the process of seed growth.

2.3. Preparation of PB nanocubes by the self-assembly approach

The reactants for PB formation were prepared as follows: solution A, 0.01 M $K_4[Fe(CN)_6]$+0.1 M KCl+0.1 M HCl; solution B, 0.01 M $FeCl_3$+0.1 M KCl+0.1 M HCl. Preparation of each self-assembled layer required four dipping operations of the electrode in solution A, deionized water, solution B and deionized water in turn. The dipping time in solutions A and B was 60 s each time, and the three washing steps in deionized water were set at 15 s each time. Normally, multiple layers were used for preparation of the film.

2.4. Immobilization of glucose oxidase

The enzyme solution was prepared by mixing 5 mg glucose, 5 μL 2% glutaraldehyde solution and 2 mg graphene to 500 μL with phosphate buffer (PBS) solution. For immobilization, 5 μL enzyme solution was placed on each electrode surface and stored at 4 °C for use.

3. Results and discussion

3.1. Formation of the PB seed layer

The PB seed layer requires two functions for biosensor fabrication: one is the provision of growth cores for further crystallization, and the other is the transmission medium for the produced electrons. Seed crystals should be small and have a uniform distribution to provide sufficient space for the regular growth of each PB crystal. The seed layer was formed *in-situ* on the Au electrode by ultrasonic force in $K_4[Fe(CN)_6]$ solution, which relied on the decomposition of $[Fe(CN)_6]^{4-}$ in the acidic solution to produce unbound iron ions [19]. The dissociation of Fe^{2+} from $[Fe(CN)_6]^{4-}$ is usually very slow, but the oxidation of Fe^{2+} is much faster. In terms of reaction kinetics, high molarity of the reactant can accelerate the reaction rate to obtain more products. Therefore, in order to determine the preferred seed layer, the surface morphology and roughness of the electrodes prepared with different $K_4[Fe(CN)_6]$ concentrations were studied using AFM. At the same ultrasonic deposition time, lower concentrations did not easily produce PB crystals, and only a few particles were formed on the supporting electrode (Fig. 1a). When the concentration reached 10 mM, a uniform crystal layer was formed with a roughness of 153 nm (Fig. 1b). However, with further increases in $K_4[Fe(CN)_6]$ concentration, large crystals accumulated together to form an uneven surface at some sites (Fig. 1c and d).

These samples were then characterized by the CV technique to confirm the influence of the seed layer on electrochemical properties (Fig. 2). It was found that the current value of redox peaks was enhanced with increased $K_4[Fe(CN)_6]$ concentration which was attributed to the increased coverage of PB seeds. In addition, the potential difference of redox peaks, which can indicate the strength of electron transfer resistance [20], were calculated to be 41, 38, 90 and 71 mV for the electrodes prepared under 5, 10, 30 and 50 mM $K_4[Fe(CN)_6]$, respectively. When considering the above AFM characteristics, these results demonstrated that 0.01 M $K_4[Fe(CN)_6]$ reactant was the optimum condition for synthesizing a uniform PB seed layer with low electron transfer resistance for ultrasonic deposition.

3.2. The influence of self-assembled layers

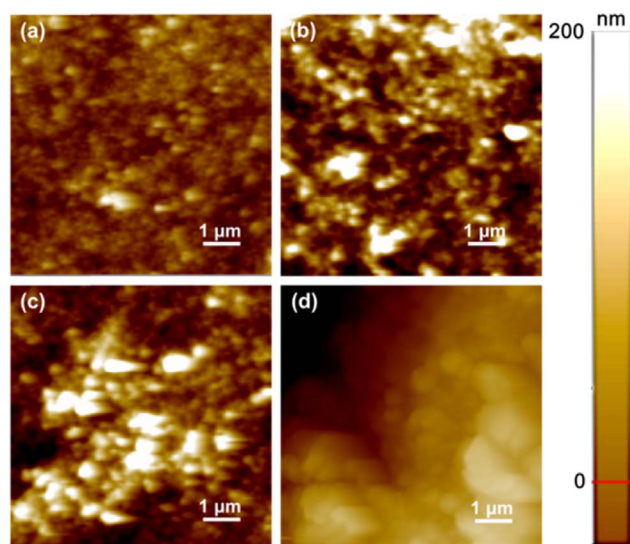Following preparation of the seed layer, further synthesis of the PB

Fig. 1. AFM images of seed layers prepared using different reactant concentrations: (a) 5 mM; (b) 10 mM; (c) 30 mM; (d) 50 mM.