

Fabrication of nanoporous thin-film working electrodes and their biosensing applications



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ABSTRACT

Electrochemical detection for point-of-care diagnostics is of great interest due to its high sensitivity, fast analysis time and ability to operate on a small scale. Herein, we report the fabrication of a nanoporous thin-film electrode and its application in the configuration of a simple and robust enzymatic biosensor. The nanoporous thin-film was formed in a planar gold electrode through an alloying/dealloying process. The nanoporous electrode has an electroactive surface area up to 40 times higher than that of a flat gold electrode of the same size. The nanoporous electrode was used as a substrate to build an enzymatic electrochemical biosensor for the detection of glucose in standard samples and control serum samples. The example glucose biosensor has a linear response up to 30 mM, with a high sensitivity of $0.50 \mu\text{A mM}^{-1} \text{mm}^{-2}$, and excellent anti-interference ability against lactate, uric acid and ascorbic acid. Abundant catalyst and enzyme were stably entrapped in the nanoporous structure, leading to high stability and reproducibility of the biosensor. Development of such nanoporous structure enables the miniaturization of high-performance electrochemical biosensors for point-of-care diagnostics or environmental field testing.

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1. Introduction

Lab-on-a-chip technology has attracted researchers from different disciplines to explore this technology for a wide range of applications (Chin et al., 2012; Ducrée et al., 2007; Huang et al., 2012; Madou et al., 2006; Whitesides, 2006). Enzymatic electrochemistry is one of the most applied detection techniques used in lab-on-a-chip devices to miniaturize chemical and biological analysis processes due to its low cost, high sensitivity, moderate power requirements, and prominent compatibility with micro-fabrication technologies (Mir et al., 2009; Vandaveer et al., 2004; Wang et al., 2001). Although the miniaturization will not alter the mechanism of biochemical reactions on electrodes, it will change fluid mechanics, molecular diffusion and surface to volume ratio in micro scale channels and will require the modification of bulk electrochemical biosensors.

All types of enzymatic electrochemical biosensors, electrodes (working electrode, reference electrode and counter electrode) perform as transducers, which convert information of a specified amount of biological analyte into an electrical signal. The biorecognition phenomenon and the redox reaction occur on the

surface of the working electrode where the catalyst layer, the enzyme layer and the semi-permeable layer are superimposed. Therefore, the working electrodes employed in microfluidic chips deserve considerable attention. To fit the electrode in the detection chamber and to minimize the disturbance to fluid movement, the thickness of the embedded planar electrode must be reduced to nanoscale and the surface area must be as small as possible. However, the miniaturization results in several implications require further improvement. For example, the miniaturization impairs the signal-to-noise ratio and the nanoscale thickness of the metal layer limits the choice of surface functionalization for subsequent coating. Because of the increasing demand for applying electrochemical sensing technique on Lab-on-a-chip devices, the electrochemical sensing surface is expected to be thinner and possess a higher signal-to-noise ratio.

Efforts have been made to enhance the sensing sensitivity through artificially enlarging the surface area of working electrodes. Recently, porous materials prepared by sol-gel methods have been developed to immobilize enzymes (Carturan et al., 1998; Liu et al., 2000). Nanostructured materials have also attracted much attention due to their inherently large surface area. For example, carbon nanotubes, which were randomly or uniformly immobilized with enzymes, were used in the configuration of biosensors (Wang, 2005). Metal nanoparticles and nanowires were also prevalent selections for substrate materials (Hrapovic et al., 2004).

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However, these matrices have limitations, including fragility, poor adhesion to the electrode surfaces and complicated fabrication procedures, which may lead to the loss of enzyme activity and poor performance in electrochemical biosensors.

Herein, a planar nanoporous thin-film working electrode was introduced for the first time. The nanoporous structure is constructed within a 100 nm thick gold (Au) electrode rather than adding another layer on top, reducing the total thickness of all layers (the metal layer, the catalyst layer, the enzyme layer and the semi-permeable layer) that stack on the electrode surfaces and improving the bonding among layers. This working electrode design was applied in the construction of planar enzymatic electrochemical biosensors. As hydrogen peroxide (H_2O_2) is a common byproduct in oxidase enzyme reactions, Prussian Blue (PB) was used as a redox mediator to enhance the selectivity of H_2O_2 detection on working electrodes (Karyakin et al., 2000). We found that the nanoporous working electrode not only possesses a large surface area, but also is an excellent substrate to hold enzymes and catalysts. In the following sections, the fabrication and characteristics of the nanoporous and planar thin-film electrodes are described, whilst the electrochemical sensing of glucose is reported as an example to demonstrate the superior performance and robustness of such biosensors.

2. Experimental section

2.1. Materials and equipment

D-(+)-glucose, uric acid, sodium L-lactate, ascorbic acid, glucose oxidase (100–250 U/mg), Nafion (5 wt%), human sera (S2257—1 ml), glucose (GO) assay kit and benzyl alcohol (anhydrous, 99.8%) were purchased from Sigma-Aldrich. Piranha solution was made using H_2O_2 (30%) and H_2SO_4 (conc.) solution in 1:3 vol/vol ratio. All processing solutions were prepared with de-ionized (DI) water from a Milli-Q system (Millipore, Bedford, MA) and all other chemicals were of analytical grade. 20 mM phosphate buffer solutions (PBS) with 0.1 M KCl were adjusted to pH 5.1. Standard samples were prepared through the dissolution of D-(+)-glucose in PBS. The samples were mutarotated overnight, forming an equilibrium mixture that consists of approximately one-third α -D-glucose and two-thirds β -D-glucose. Human serum samples were made by dissolving lyophilized sera with 1 ml DI water. A nanoporous thin-film working electrode, a Ag/AgCl reference electrode in 1 M KCl and a Pt counter electrode comprised the electrochemical sensing system. Electrochemical measurements were conducted using a potentiostat (CHI 1207A, CH Instruments, Austin, TX) at room temperature ($22 \pm 1^\circ\text{C}$). A Hitachi S-4500 field emission scanning electron microscope coupled with an energy dispersive X-ray spectroscopy (SEM/EDX) and a Veeco atomic force microscope (AFM) were employed to characterize the fine structure of electrodes. A UV–visible absorption spectrophotometer from Varian was employed to provide a reference glucose level in serum samples using a standard glucose assay kit.

2.2. Fabrication of nanoporous thin-film electrodes

Nanoporous thin-film electrodes were fabricated by selectively dissolving the less noble metal from a bimetallic alloy. The alloy was made by consecutively depositing Au and Zn (zinc), followed by thermal annealing. Prior to the metal deposition, a silicon wafer was cleaned with piranha solution at 80°C for 20 min. The pattern of the working electrode was defined by a positive photoresist Shipley 1805 on the cleaned wafer, which was then loaded into an electron beam evaporation chamber and successively deposited 5 nm Cr (chromium) and 100 nm Au, where Cr

served as an adhesive layer. Afterwards, the sample was taken out, and the lead and conductive pad were covered with a piece of tape. A 500 nm Zn Layer was deposited on top of Cr by using the sputtering technique. After a lift-off process, where the metal in the non-electrode area was removed using a PG remover (N-methyl pyrrolidinone), the sample was annealed in benzyl alcohol at 100°C for 60 min and rinsed with isopropanol (IPA) and DI water. Finally, the alloyed samples were dipped into 50% HNO_3 for 2 min to chemically dissolve the Zn.

2.3. Deposition of Prussian blue, enzyme and nafion

Electrodeposition of PB was accomplished using cyclic voltammetry (CV) (Karyakin et al., 2000). Before the electrodeposition, the nanoporous electrodes were electrochemically cleaned and activated in 0.5 M sulfuric acid by cycling between 1.5 V and -0.5 V at a sweep rate of 40 mV s^{-1} until a stable voltammogram was obtained. The solution for the electrodeposition of PB comprised 1.5 mM $\text{K}_3[\text{Fe}(\text{CN})_6]$, 2 mM $\text{Fe}(\text{NO}_3)_3$, 1 mM HCl and 0.1 M KCl. The electrode potential was cycled 30 times from 0.6 V to -0.2 V at a sweep rate of 50 mV s^{-1} . Subsequently, the electrodes were rinsed with DI water and activated in 0.1 M KCl+0.1 M HCl by cycling the applied potential between 0.5 V and -0.2 V with scan rate of 50 mV s^{-1} until stable CV curves were obtained.

Glucose oxidase (GOD) was immobilized onto the PB-modified electrode surface by physical adsorption. The lyophilized GOD powder was dissolved in PBS to form an enzyme solution with a concentration of 20 mg/mL. 5 μL of the enzyme solution was dropped onto the electrode surface and dried in a fridge (4°C). Nafion solution of 5% was added on top of the enzyme layer to protect against the leakage of the enzymes and reduce the influx of glucose. The Nafion solution dried in the fridge as well. With these steps, the biosensor was completely built and ready to be used immediately or stored in PBS in the fridge.

3. Results and discussion

3.1. Characterization of nanoporous thin-film electrodes

The formation of nanoporous structures on the working electrode layer is the key to their superior performance. The sputtered Zn layer covering the Au layer was shiny and gray before annealing. The sample turned dark gray at the end of the annealing process, which indicated the successful alloying. Annealing was conducted in benzyl alcohol to prevent the oxidation of Zn to ZnO, which stops the alloying process. In the following dealloying process, the Zn component was dissolved by nitric acid and the remaining Au component gradually diffused to the surface to form bundles, which could further develop into an interconnected porous network. The golden color of the surface turned into a dim copper-like color (ESI: Fig. S1) at the moment the nanoporous structure was formed. As shown in Fig. 1, the smooth Au film electrode (Fig. 1a) turned into nanoporous structures (Fig. 1b) after the dealloying treatment. The nanoscaled ligaments and nanopores are continuously connected to form a honeycomb structure, which not only enlarges surface area, but also is an excellent substrate for electrode modification. From AFM images (Fig. 1c and d), we can see that the roughness was increased from 2 nm for the planar Au to 20 nm for the nanoporous Au. Further, all the Zn atoms should be removed, otherwise, the electrochemical signal would be disturbed by the oxidation of leftover Zn. In order to remove Zn without destroying the ultrathin nanoporous Au film, EDX analysis was utilized to inspect the composition of the film and

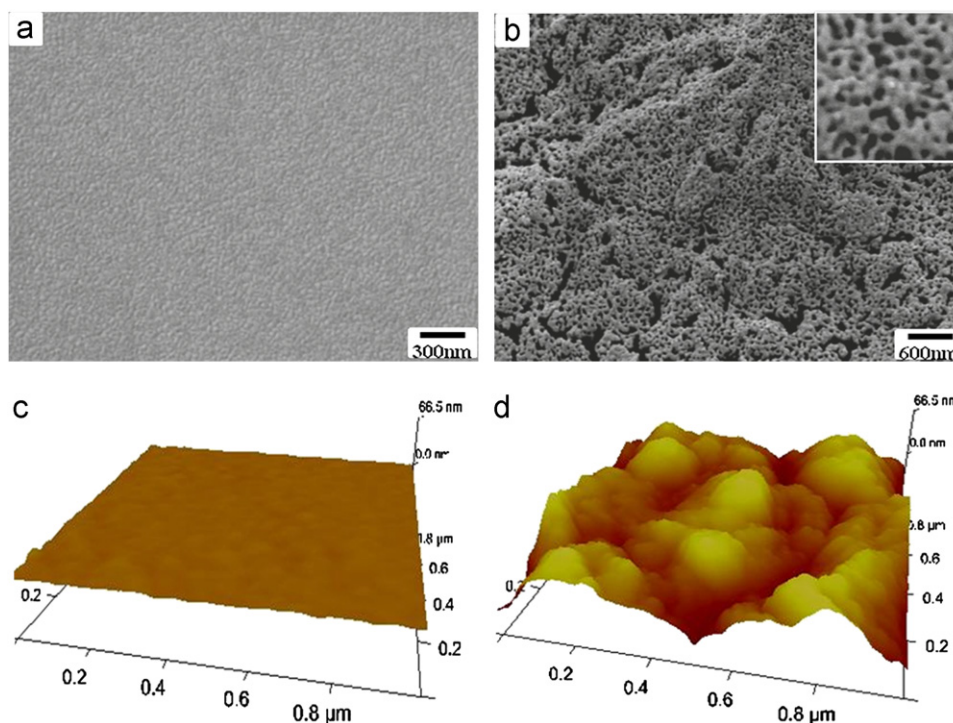


Fig. 1. SEM and AFM images show (a, c) the smooth and flat surface of a Au electrode before nanoporous treatment; (b, d) the nanoporous surface of a Au electrode after nanoporous treatment.

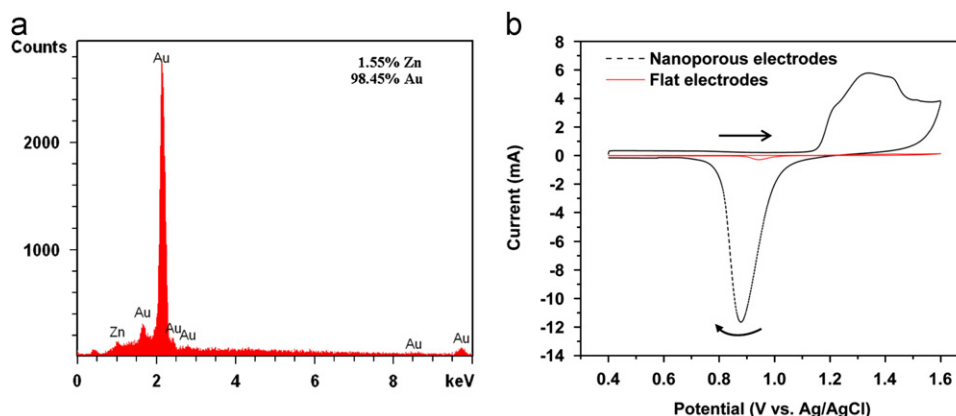


Fig. 2. (a) EDX spectrum of a nanoporous electrode immersed in 50% HNO_3 solution for 120 s. (b) Cyclic voltammograms recorded in 0.5 M H_2SO_4 for a flat Au electrode and nanoporous Au electrode show that the cathodic peak of the non-porous Au electrode is only one fortieth of that of the nanoporous Au electrode.

optimize the immersion time of samples in the HNO_3 solution. The EDX spectrum in Fig. 2a shows that only 1.55% Zn remained in the nanoporous films after immersing the sample in the HNO_3 solution for 120 s. The concentration of Zn changed little by further increasing the etching time in the HNO_3 solution. Explanation for this phenomenon is that the porous film containing a small amount of Zn possessed similar corrosion behavior as that of pure Au. Therefore, Zn atoms would not be selectively etched to the extent of 100% without destroying the porous structure (Jia et al., 2007).

It is known that nanoporous electrodes possess a larger surface area than flat electrodes. Among various determining methods, the method based on the reduction of gold oxide, the charge of which is proportional to the active surface area, appears to be straightforward (Trasatti and Petrii, 1991). Cyclic voltammograms of a flat Au electrode and a nanoporous Au electrode were

recorded in a 0.5 M H_2SO_4 solution and are compared in Fig. 2b. These voltammograms reveal that the formation of gold oxide results in the peak of the current at the anodic scan, while the reduction of gold oxide induces the cathodic peak at 0.88 V. As the nanoporous Au electrode possesses a larger surface area, the corresponding cathodic current appears up to 40 times higher than the flat non-porous Au electrode.

The nanoporous structure not only has a larger surface area, but also serves as an excellent substrate for electrode modification. The electrodeposited microcrystalline PB powder was firmly and evenly entrapped and distributed on the nanoporous surface (Fig. 3a), but the PB film electrodeposited on the flat Au electrode was lumpy and full of cracks (Fig. 3b). When the electrodes were cycled 20 times in the PBS from 0.5 V to -0.2 V at a scan rate of 50 mV s^{-1} , majority of PB powder remained on the nanoporous surface (Fig. 3c), but in contrast a large amount of crystallized PB

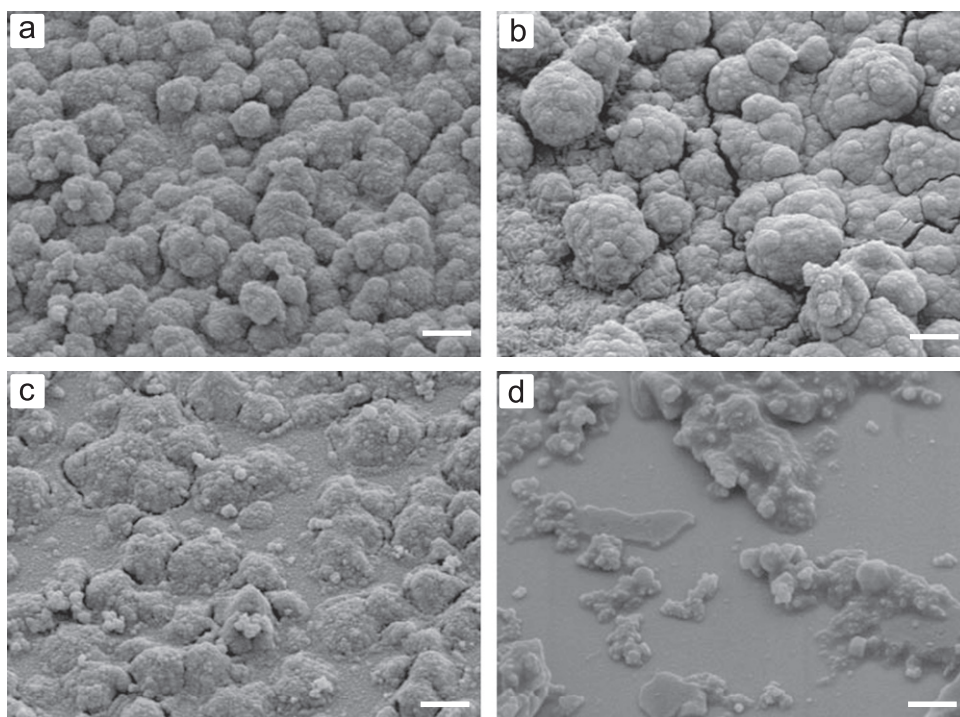


Fig. 3. SEM images of uniform and compact PB film deposited on a nanoporous electrode (a) and subjected to 20 cycles of potential scan in a PBS (c); lumpy and loose PB film deposited on a flat electrode (b) and subjected to 20 cycles of potential scan in a PBS (d). All the scale bars are 1 μm .

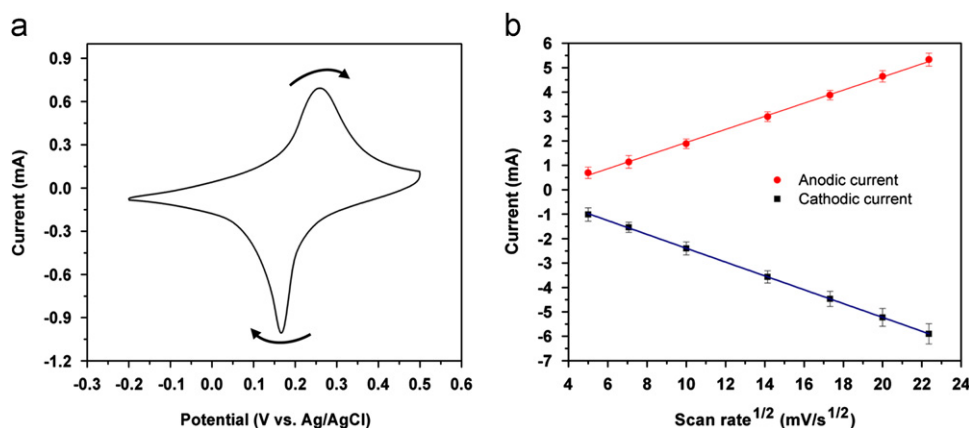
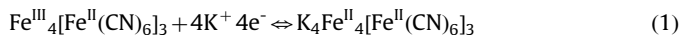


Fig. 4. (a) Cyclic voltammogram of the PB-modified nanoporous electrodes at a scan rate of 25 mV s^{-1} in the PBS; (b) Plot of anodic and cathodic currents versus the square root of the scan rates. The regression equation of the linear fit to anodic response is: $y = -0.74 + 0.26x$ ($R^2 = 0.9984$, $n = 5$), and to cathodic response is: $y = 0.44 - 0.28x$ ($R^2 = 0.9998$, $n = 5$).

was stripped off from the flat electrode surface (Fig. 3d). This merit of the nanoporous electrode significantly increases the stability and reproducibility of the biosensor.

Before detecting biological samples, the nanoporous electrode with the PB layer was characterized using cyclic voltammetry in the PBS. The PB films were characterized with a benchmark compound $\text{Fe}^{3+}/\text{Fe}^{2+}$ redox couple and showed electrochemical behavior (cyclic voltammetry) as follows:



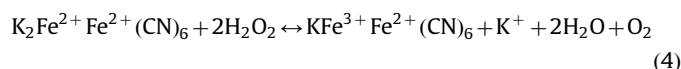
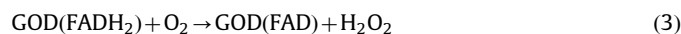
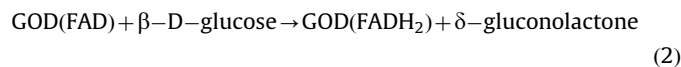
In a forward scan from 0.5 V to -0.2 V with scan rate of 25 mV s^{-1} , ferricyanide was reduced to ferrocyanide and reached a cathodic peak current at 0.16 V . In a return potential scan, a reversed electron transfer reaction occurred and reached an anodic peak current at 0.26 V (Fig. 4a). The peak values of the forward and reversed reactions are linearly proportional to the square root of the scan rates in the range from 25 to 500 mV s^{-1}

(Fig. 4b). The linearity implies that the rate of the redox reactions is controlled by the diffusion of potassium ions, which produces a concentration gradient of the electroactive species in the PB lattice on the electrode. The rate of the electron transfer step is fast compared to the rate at which potassium ions diffuse from the bulk solution to the electrode surface, so the peak current, i_p , is governed by the Randle–Sevcik relationship (Dungchai et al. 2009; Zakharchuk et al. 1995).

3.2. Calibration of the biosensor

The mechanism of the biosensor involves the electrochemical detection of H_2O_2 generated by the analyte in the enzymatic reaction. Here, we take the specific biorecognition of glucose by the enzyme glucose oxidase (GOD) as an example. GOD, containing two molecules of the cofactor flavine adenine dinucleotide (FAD), is a homodimer with a molecular weight of 160 kD . The

sensing process can be elaborated as follows: glucose diffuses from the bulk solution to the GOD layer where it is converted into gluconolactone under the reduction of the FAD prosthetic group (Eq. (2)). The cofactor is oxidized back to its original state, whereby two electrons and two protons are transferred to molecular oxygen, generating H_2O_2 (Eq. (3)). The H_2O_2 , which is proportional to the concentration of glucose (Ricci and Palleschi, 2005), then catalytically reacts with the Prussian white (PW) by oxidizing PW to PB and PB itself is, in turn, electrochemically reduced back to PW under the applied potential on the surface of the working electrode (Eq. (4)).



The biosensors were first calibrated with standard glucose samples using chronoamperometry. In the chronoamperometric measurement, the potential of the working electrode is stepped, so that the Faradic current generated at the electrode decays as a function of time. The Faradic current, due to the electron transfer, is proportional to the concentration of analyte, and can be described by the Cottrell equation (for planar electrodes) (Bard and Faulkner, 2001)

$$i = \frac{nFAC^0D^{1/2}}{(\pi t)^{1/2}} \quad (5)$$

where i is the current, n is the number of electrons, F is Faraday's constant, A is the area of the electrode, t is the time, C^0 and D are the initial concentration and the diffusion coefficient of the target analyte, respectively.

The potential of 0 V (versus Ag/AgCl) has been optimized for cathodic reduction of hydrogen peroxide assisted by PB (Dungchai et al., 2009). Therefore, chronoamperometric measurements were carried out on glucose standard samples with concentration ranging from 0 mM to 40 mM using 0 V step potential. In Fig. 5a, the Faradic current generated right after a large capacitive current, decays as $t^{-1/2}$ until its steady state. The steady-state currents at 100 s were picked up to draw a calibration curve for this biosensor. The mean and the relative standard deviation were generated with a group of data from five biosensors. The calibration curve (squares) in Fig. 5b demonstrates the linear relationship between currents and analytes with a

concentration of 0–30 mM ($R^2 = 0.9971$, $n = 5$). The signal for 40 mM is out of the linear range. This linear range has been able to cover the ranges of normal human blood glucose of 3.5–6.1 mM (in whole blood) and abnormal glucose levels (20–30 mM). The sensitivity of the glucose analysis is calculated to be $0.50 \mu\text{A mM}^{-1} \text{mm}^{-2}$, higher than the value ($0.43 \mu\text{A mM}^{-1} \text{mm}^{-2}$) achieved by the electrochemical system built on the porous paper (Nie et al., 2009). The limit of detection (LOD), calculated as the concentrations which produced a signal of three times greater amplitude than noise ($S/N = 3$), is about 0.3 mM, providing better performance compared to the conventional colorimetric methods (LOD, 0.5 mM) and glucometers (LOD, 1 mM) (Hönes et al., 2008; Martinez et al., 2008). The conventional flat electrodes with immobilized enzymes were also measured using the same condition, and the data were plotted in Fig. 5b (circles). The result of the comparisons clearly shows that the nanoporous biosensor possesses higher sensitivity than conventional biosensors for glucose detection. Since the detection of glucose is actually the detection of H_2O_2 in the biosensors, the nanoporous Au electrodes also have high sensitivity for H_2O_2 detection. The increase of the electroactive surface area (A) results in the sensitivity enhancement, which is supported by the Cottrell equation.

3.3. Analytical performance

Human serum samples were assayed in order to validate that the nanoporous thin-film electrochemical system is beneficial to the practical usage of the glucose biosensor. The glucose concentrations of these serum samples were measured based on a spectrophotometric method using a standard test kit, and used as reference values. The steady-state currents of serum samples were then collected in the same condition as in the calibration step, and the glucose concentration was calculated using the previous calibration curve. The measured value with the spectrophotometric method was 5.43 ± 0.08 mM, whilst it was 5.51 ± 0.03 mM with the electrochemical method using the biosensor. The approximation of these two values proves the practical utility of the proposed biosensor. The robustness of the biosensor was further proved by showing the recovery of glucose concentration by a standard addition method. The recovery rates were in the range of 96–103% for human serum samples spiked with 0.5 mM glucose. In addition, the biosensor retained its enzyme bioactivity and still had about 80% of its initial sensitivity after 10 days stored in PBS at 4 °C.

As an inherent merit of using PB, the biosensor showed little interference to the electroactive interfering compounds commonly

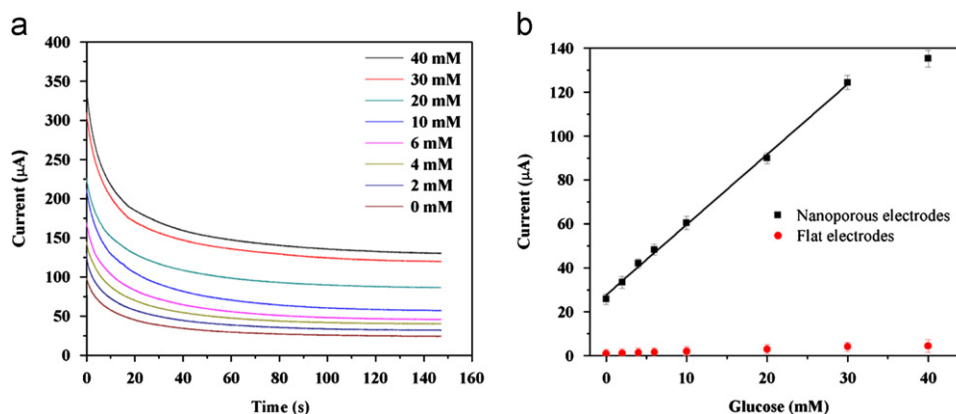


Fig. 5. (a) i - t curves of glucose with concentration from 0 mM to 40 mM. The disc working electrode with a diameter of 3 mm was used to record data at 0 V versus Ag/AgCl. (b) Currents of chronoamperometric curves at 100 s plotted as a function of the concentration of glucose. Square dots and circular dots represent data from nanoporous biosensors and conventional biosensors, respectively. The regression equation for the linear fit is: $y = 25.71 + 3.19x$ ($R^2 = 0.9971$, $n = 5$).

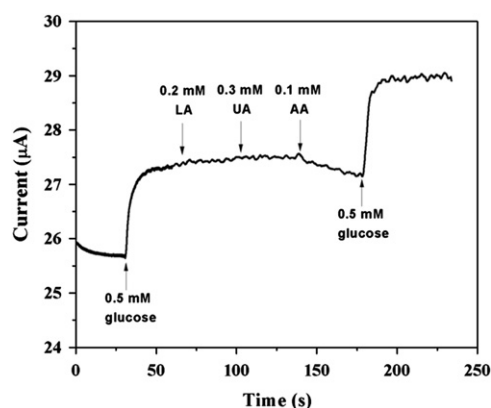


Fig. 6. Time course amperometric response to successive adding of glucose and interferents. The experiment was conducted in PBS at 0 V versus Ag/AgCl under stirring. Under this condition, addition of 0.2 mM lactate (LA), 0.3 mM uric acid (UA) and 0.1 mM ascorbic acid (AA) introduced 0.7% and 0.3% increasing, and 1.5% decreasing, respectively.

present in physiological samples, including lactate (LA), uric acid (UA) and ascorbic acid (AA) due to the low operating potential. The utilization of Nafion as an outermost layer on the electrode surface further ensures its sensitivity to glucose by dramatically reducing the diffusion of interfering compounds to the electrode surface. The normal concentrations of LA, UA and AA in serum are 0.2 mM, 0.3 mM and 0.1 mM, respectively. The amperometric response of the biosensor to successive additions of glucose (0.5 mM) and the interferents into the stirring PBS is shown in Fig. 6. The current rose only 0.7% and 0.3% during the additions of 0.2 mM LA and 0.3 mM UA, respectively, while the presence of 0.1 mM AA slightly depressed the current by about 1.5%. Therefore, the measured values of glucose concentration in addition to the LA, UA and AA are 0.504 mM, 0.505 mM and 0.498 mM, respectively. The addition of 0.5 mM glucose before and after the existence of those interferents produced an equivalent response, so the summation of interferents did not denature the biosensor. These results demonstrated the excellent anti-interference ability of the biosensor.

3.4. Discussion

The fabrication and application of nanoporous structures have been reported in bulk electrodes by other researchers, but the fabrication of nanopores in a thin-film planar electrode and its application in electrochemical detection are conducted here for the first time. The results presented above show that the nanoporous electrode is of uniform pore structure, large electroactive surface area, and high electrical conductivity. It is these qualities that make it a robust platform for the immobilization of catalysts and enzymes and provides excellent performance in biosensing trials.

Modification of electrodes through layer by layer assembly of organic and inorganic material possesses an inherent adhesion problem between the assembled layers and the flat electrode surface, and so the risk of the catalysts/enzymes being washed out of the modified electrodes or leached into the solution is increased, which impairs the stability and performance of the electrode system. It is also known that the PW (the reduced form of PB) at potentials around 0.0 V is unstable and hydroxyl ions produced by the reduction of H_2O_2 in neutral media make the inorganic polycrystal partially soluble, so the electrodeposited PB could easily strip off from the flat electrode surface, leading to the loss of the electrocatalyst (Itaya et al., 1984; Karyakin et al., 1999). In this experiment, blue pigment could be observed by naked eyes due to the detachment of PB film from the flat electrode surface

whether using the cyclic voltammetric deposition technique or the constant potential deposition technique. However, this rarely happens on nanoporous electrodes, as most of the deposited PB film is firmly entrapped in the nanopores.

The nanoporous structure can not only firmly hold PB particles during the electrode modification process (Fig. 3), but also benefits from retaining enzymes when the biosensor is integrated in the microfluidic system. Sample flow rates in microfluidic system normally range from nL s^{-1} to liter s^{-1} (Madou et al., 2006). When it flows across the conventional working electrodes, the sample fluid will continuously bring away the portions of immobilized enzymes, resulting in a decrease of the stability of biosensors. As shown in Fig. S2, the nanoporous structure serves as a better platform to prevent enzyme leakage under a sample flow rate of $30 \mu\text{L s}^{-1}$.

The advantage of the nanoporous electrode is also demonstrated by its superior performance after the enzyme and nafion are entrapped in electrodes. First, the nonporous structure possesses a larger surface area and more active sites, which results in an increase of steady-state Faradic current, compared with the flat electrode surface. Second, immobilization methods based on chemical modification can change the Michaelis–Menten constant of the enzymes because of the steric and conformational alterations (Guisan, 2006). Therefore, physical adsorption and entrapment is a better method to retain enzyme activity. Third, nanoporous metals can enhance the electrochemical transduction of redox enzymes. The continuity of metallic nanoscaled ligaments and nanoporous channels contributes to good electrical conductivity of nanoporous metals. The catalyst and the enzyme are well-dispersed and entrapped in the porous matrix, so that these form a conducting network aiding charge transfer through the film. Therefore, the biosensor has a higher response to the target analyte due to the high activity of electrocatalysis (Yu et al., 2007; Zhang et al., 2007).

4. Conclusions

We have successfully fabricated nanoporous structures directly on a planar thin-film Au electrode, and applied them to configure an enzymatic electrochemical biosensor. Experimental results show that the layer of nanosized metallic ligaments and nanopores complexes possess 40 times more active surface area than a flat layer and can act as an ideal platform for the immobilization of catalysts and enzymes, with enhanced charge transport and bioelectrocatalytic properties, yielding high electrochemical responses. Use of this biosensor in the detection of glucose shows a wide linear range (up to 30 mM), high sensitivity ($0.50 \mu\text{A mM}^{-1} \text{mm}^{-2}$) and low interference. This work shows great promise for designing on-chip electrochemical biosensors used in point-of-care diagnostics.

Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.bios.2012.10.003>.

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