

Fabrication and Electrochemical Characterization of Nanoporous Silicon Electrode for Amperometric Urea Biosensor

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We describe a new type of biosensor that employs a modified gold electrode based on nanoporous silicon (NPSi) for the electrochemical detection of urea. Urease (Urs) was covalently immobilized onto an Au/NPSi electrode functionalized with 3-mercaptopropionic acid (3-MPA). Amperometric calibration curves for both NPSi and planar silicon (PLSi)-based urea sensitive electrodes were compared in the range of 0.3 to 4.5 mM urea concentrations. The Michaelis–Menten constant (K_m) was determined using the amperometric method. The electrochemical active area (A_{ea}) of the 3-MPA/Au/NPSi electrode was evaluated using cyclic voltammetry (CV) and the result was compared with the 3-MPA/Au/PLSi electrode. Measured sensitivity of the Urs/SAMs/Au/NPSi electrode is ca. $2.05 \mu\text{A mM}^{-1} \text{cm}^{-2}$ and that of the Urs/SAMs/Au/PLSi electrode is ca. $1.10 \mu\text{A mM}^{-1} \text{cm}^{-2}$. About 1.8 times of sensitivity increase is obtained in the Au/NPSi electrode.

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1. Introduction

Nanoscale electrodes based on porous materials have been observed in many applications, such as photochemical solar cells,^{1,2} energy storage,^{3,4} chemical and biological sensors.^{5–8} For biosensor applications, miniaturization into a microsensor with flat surface is very difficult, since the scaling down of the active sensor area for enzymes or other biomolecules immobilization is accompanied by a proportional decrease of the measured signal to small absolute signal values.^{9,10} In order to increase the signal, the most commonly used methods are modification of gold electrode as the substrate immobilized biomolecules. Although many strategies for highly sensitive and good stabilized biosensors have been developed, achieving the application fields of biosensor is still a very difficult task because the progress of porous metal fabrication is tedious and involved multiple steps that strongly acidic, alkaline, or ionic electrolyte solutions are used in the typical electrochemical process.

Nanoporous silicon (NPSi) is a unique and diverse material that has several features that make it especially attractive for biosensors, including a three-dimensional architecture, simple and inexpensive fabrication techniques, tuneable pore size and porosity, and suitability for integration with silicon electronics.¹¹ NPSi electrode has a large and highly reactive surface area compared with the same size of planar electrode, which enables more effective capture and detection of molecules than planar electrode.¹² Moreover, this principal property of the porous electrode makes it possible to the rapid and efficient exchange of ions between porous electrode and aqueous solution. NPSi electrodes are mostly employed in the optical biosensors due to the high quality optical detection based on changes of properties such as photoluminescence or reflectance, on exposure to the gas or liquid solution.^{13–15} NPSi electrodes are also found several electrochemical biosensors and often favored over planar electrodes for many reasons.^{16–18} For instance, when only a liquid phase contacts the electrode, NPSi electrodes are used to increase the surface area for charge transfer, thereby reducing the electrode overpotential for H_2O_2 oxidation and reduction reaction. Moreover,

surface structure of the PLSi substrate is favorable since the diffusion of analytes in and out of pores should easily be possible and additional metallic thin layers can be deposited onto the pore walls by the physical vapor deposition.

The metabolic function of the kidney is reflected in the concentration of organic compounds, such as urea, in blood or urine. Therefore monitoring of urea levels is highly significant for an early diagnosis, many researchers have been reported for the fabrication of urea sensors based on the various electrochemical technologies. The first potentiometric urea sensor was reported in 1969 by Guilbault who immobilized the urease (Urs) in a polyacrylamide gel held over the surface of a monovalent cation electrode.¹⁹ However, potentiometric methods in principle suffer from the disadvantages of low accuracy and reproducibility.^{20,21} In order to avoid these problems, Impedance methods was reported based on measuring the pH change in capacitance induced by Urs reaction.^{22,23} Also, Mizutani *et al.* proposed a voltammetric method by utilizing a layer of lipid attached Urs on the hydroquinone (H_2Q) monolayer-modified gold.²⁴ This method requires the addition of electroactive species in the test solution as a pH-indicator, which is inconvenient for the operation of the sensor.

In an attempt to develop enzyme biosensor which is enhanced in amperometric sensitivity compared with gold electrode on planar silicon (PLSi) substrate, we investigated the possibility of utilizing a modified gold electrode based on NPSi, namely, the urea sensitive electrode for the preparation of enzyme electrode. Also, this paper reports the electrochemical characteristics of 3-mercaptopropionic acid (3-MPA)/Au/NPSi based urea sensor as a basic experiments applicable to micro-biosensor. It was shown that the Michaelis–Menten constant (K_m) of the immobilized Urs on 3-MPA/Au/NPSi was considerably smaller than that on 3-MPA/Au/PLSi. Electrochemical active area (A_{ea}) of the 3-MPA/Au/NPSi electrode was evaluated by applying the Randles-Sevcik equation and the result was also compared with the 3-MPA/Au/PLSi electrode.

2. Experimental Procedure

2.1 Instruments

Cyclic voltammetry (CV) and amperometric response measurements of the electrodes were performed using an

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electrochemical workstation composed of a multi-potentiostat/galvanostat (Bio-Logic Science Instruments VMP), and IBM compatible personal computer (PC), an N₂ gas bubbling/purging system, and a Teflon electrochemical cell. A conventional three-electrode system was used in all of our measurements. PLSi and NPSi electrodes were used as working electrodes, an Ag/AgCl electrode with saturated KCl internal solution was used as a reference electrode, and a spiral platinum wire acted as a counter electrode.

2.2 NPSi preparation

A boron-doped, (100)-oriented, p-type silicon wafer with a 1 to 10 Ω-cm resistivity was used as an electrode substrate to form an electrochemically-etched NPSi layer on the surface. The electrolyte solution was composed of HF (49%) : C₂H₂OH (95%) : deionized water = 1 : 2 : 1 by volume, and a current density of -7 mA/cm^2 was maintained in a specially designed Teflon cell to form a uniform NPSi layer. The NPSi substrate was oxidized at 400 °C in dry oxygen for 1 h, followed by annealing at the same temperature in nitrogen for 15 min. The Ti (20 nm) and Au (250 nm) layers were deposited by chemical vapor deposition in a vacuum at a base pressure of 2×10^{-5} mbar and a temperature of 100 °C.

2.3 Urs immobilization

Urs was immobilized onto the 3-MPA/Au/NPSi electrode by covalent attachment.²⁵ For the covalent immobilization, the gold electrode modified with a self-assembled monolayer containing terminal carboxylic groups was treated with a stirred 0.1 M sodium phosphate buffer solution at pH 7.4, containing *N*-hydroxysulfosuccinimide (NHS; 3 mM) and 1-ethyl-3-[3-(dimethylamino)-propyl] carbodiimide (EDC; 100 mM) at room temperature. After a reaction time of 3 h, the electrode was rinsed with phosphate buffer and immediately placed in a stirred 5 mg/cm³ Urs solution in 0.1 M sodium phosphate buffer at pH 7.0. This step was allowed to continue overnight at room temperature. The electrode was then thoroughly rinsed with the following sequence of liquids: pH 7.0 phosphate buffer solution, and 1 M NaCl, Milli-Q Plus water. Finally the electrode was blown dry with nitrogen.

3. Results and Discussion

The implementation of the proposed structure is shown schematically in Fig. 1(a). The sensing electrode has four layers composed of the Urs, 3-MPA, Au, Ti, and the NPSi substrate from the top to bottom layers. A round-sensing electrode (radius; 0.25 mm, area; 0.19 cm²) was used as a working electrode [Fig. 1(b)]. Figure 1(c) shows a scanning electron microscope (SEM) image of a NPSi-based gold electrode. The pore diameter and depth are approximately 100 and 250 nm, respectively.

The electrochemical active area (A_{ea}) of the NPSi-based gold electrode was determined from the cyclic voltammetric result of redox reactions of K₃Fe(CN)₆ on the electrode surface and compared with the A_{ea} of the PLSi-based gold thin-film substrate.²⁶ Electrochemical properties of the working electrodes (Au/PLSi, Au/NPSi) used to predict the effect of electrode geometry on cyclic voltammetric response are shown in Fig. 2. CVs were obtained by the

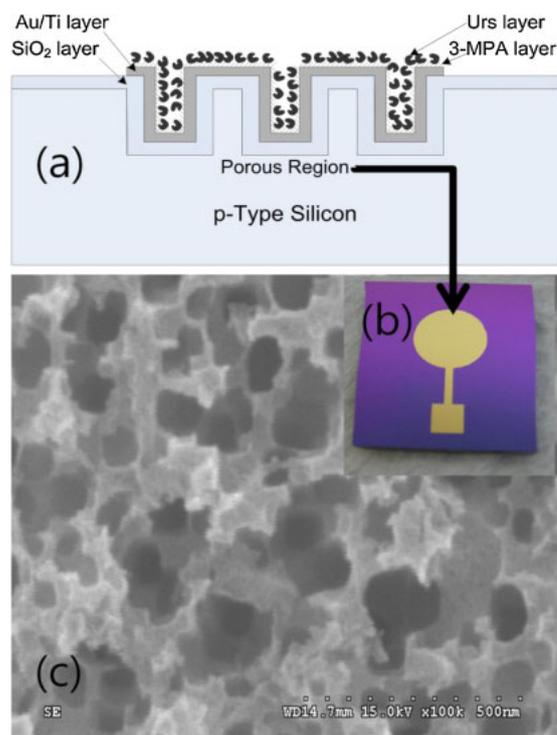


Fig. 1. (Color online) (a) Schematic illustration, (b) photograph, and (c) SEM image of NPSi-based gold electrode. The thickness of the Ti and Au thin-film layers is 20 and 250 nm, respectively.

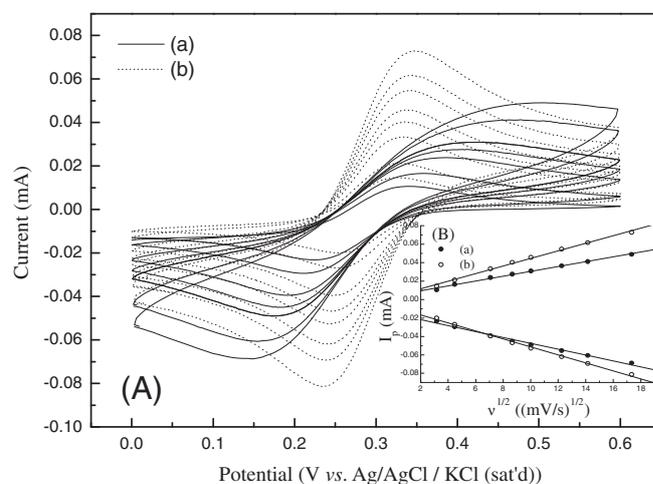


Fig. 2. Determination of A_{ea} . (A) CV diagrams of Au/PLSi (solid line) and Au/NPSi (dotted line) in 3 M KCl solution containing 10 mM K₃Fe(CN)₆ at different scan rates: 10, 20, 50, 75, 100, 150, 200, and 300 mV/s. (B) The plot of I_p versus $v^{1/2}$ for each substrate: (a) Au/PLSi (slope: 0.00264); (b) Au/NPSi (slope: 0.00412).

redox reactions of $[\text{Fe}(\text{CN})_6]^{3-}/[\text{Fe}(\text{CN})_6]^{4-}$ on the Au/NPSi and the Au/PLSi at various scan rates (10 to 300 mV/s). The inner CVs, had the lowest peak currents and represent the slowest scan rate. The applied potential ranged from 0 to 0.6 V versus Ag/AgCl with a Pt counter electrode. Figure 2(A) shows that electrode porosity caused the peak current to increase and the peak-to-peak separation to decrease. The higher scan rates of the thickness of the diffusion layer become the smaller diffusional fluxes to the bottom of the electrode and to the top do not interfere with

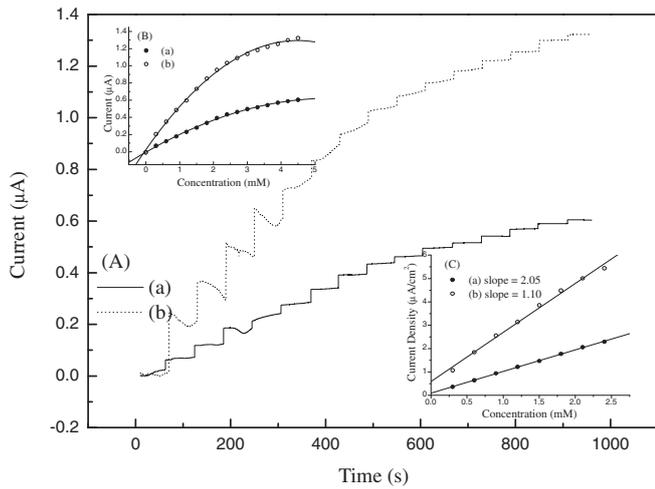
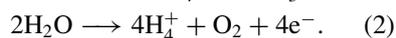
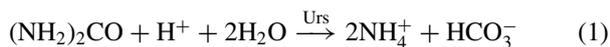


Fig. 3. Amperometric responses of the urea-sensitive electrode (A). Inset plot of amperometric current versus urea concentration (B), and linear calibration curves (C) for determination of sensitivity: (a) Urs/3-MPA/Au/PLSi ($1.10 \mu\text{A mM}^{-1} \text{cm}^{-2}$), and (b) Urs/3-MPA/Au/NPSi ($2.05 \mu\text{A mM}^{-1} \text{cm}^{-2}$). Operational potential was 0.6 V versus Ag/AgCl reference electrode.

each other, in other words the efficiency of the diffusional mass transport increased. Figure 2(B) shows that the I_p versus $v^{1/2}$ curves are linear, and the electrode reactions occurring on both electrodes were nearly completely reversible. We conclude that the mass transfer phenomenon occurred in the double layer region. According to Randles' equation, the slope in the plot of I_p versus $v^{1/2}$ is directly related to A_{ea} ; i.e., the slope ratio equals the corresponding A_{ea} ratio and directly affects the sensitivity. In this case, the A_{ea} ratio was 1.56, which means that the sensitivity of the PLSi-based sensing electrode will increase by a factor of 1.56.

Figure 3 shows a typical amperometric response for successive addition of 0.3 mM urea in a range of 0.3 to 4.5 mM at a constant potential of 0.6 V. The general principle for fabricated urea biosensor is based on immobilization of Urs onto a membrane or support in which urea is catalytically converted into ammonium and bicarbonate ions. As can be seen in eq. (1),²⁷ a urea-hydrolysis reaction induces proton consumption to increase the pH value. At this condition, an anodic current on account of the water dissociation flows as is shown in eq. (2). Where the H^+ is produced, the pH shift is compensated. The magnitude of this anodic current could be used as monitor to determine the urea concentration.



As shown in Fig. 3(A), a well-defined current was proportional to the urea concentration for (a) the Urs/3-MPA/Au/PLSi, and (b) the Urs/3-MPA/Au/NPSi. Based on the amperometric response, calibration curves were obtained in as shown Fig. 3(B) for each injection of urea solution. The current was linearly proportional to the urea concentrations in a range of 0.3 to 2.4 mM with a correlation coefficient of 0.998 and the slopes for (a) and (b) were 1.10 and $2.05 \mu\text{A mM}^{-1} \text{cm}^{-2}$, respectively. The Urs/3-MPA/Au/NPSi showed 1.8 times greater enhanced sensitivity

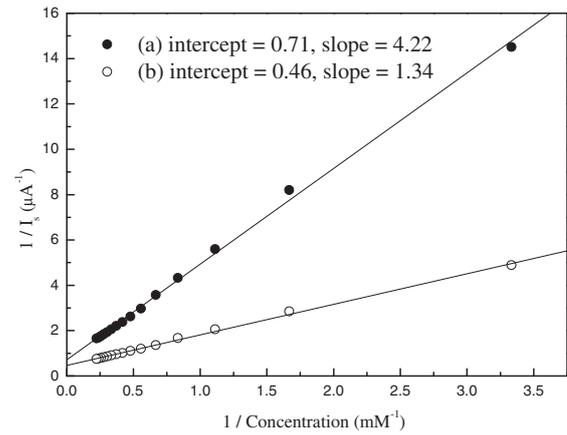


Fig. 4. Linear representation of Michaelis–Menten equation for (a) Urs/3-MPA/Au/PLSi ($K_m = 5.94 \text{ mM}$) and (b) Urs/3-MPA/Au/NPSi ($K_m = 2.91 \text{ mM}$).

compared to the Urs/3-MPA/Au/PLSi. This result is in agreement with the CV characteristics shown in Fig. 2 for both substrates.

The apparent Michaelis–Menten constant (K_m) can be calculated for an immobilized enzyme by amperometric methods,²⁸ which can be estimated from the Lineweaver–Burk equation:

$$\frac{1}{I_s} = \frac{K_m}{I_{\text{max}}} \frac{1}{C} + \frac{1}{I_{\text{max}}},$$

where I_s is the steady-state current after the addition of substrate, C is the bulk concentration of the urea, and I_{max} is the maximum current obtained using the Lineweaver–Burk plot.²⁹ The K_m value was determined by analysis of the slope and intercept of the plot of the reciprocals of the steady-state current versus urea concentration (Fig. 4). The K_m value of the Urs/3-MPA/Au/NPSi was 2.91 mM, which was lower than that of the 5.94 mM at Urs/3-MPA/Au/PLSi. The smaller K_m value implies that the immobilized Urs possessed higher enzymatic activity with respect to urea.³⁰ The mass transport of analytes and products was rapid in the meantime. The immobilization of Urs onto Au/NPSi may make a feasible microenvironment for the enzymes and affect the intrinsic properties of enzymes, which favors the proposed Urs/3-MPA/Au/NPSi to exhibit a high affinity to urea. In addition, the proposed porous structure of the electrode could improve the mass transport and enhance enzyme utilization.

4. Conclusions

The use of NPSi as a sublayer of modified gold electrode provides the opportunity for simple method to prepare the nanoporous gold electrode and high sensitivity to compare with PLSi substrate. Measured sensitivity of the Urs/3-MPA/Au/NPSi electrode was $2.05 \mu\text{A mM}^{-1} \text{cm}^{-2}$, and that of the Urs/SAMs/Au/PLSi electrode was $1.10 \mu\text{A mM}^{-1} \text{cm}^{-2}$ in the linear range of 0.3 to 2.4 mM urea concentrations. A significant increase in sensitivity was achieved with the platinumized biosensor due to the increase in the A_{ea} of the electrode's surface. In a general biosensor, the amount of enzyme immobilized on the electrode's surface

may be dependent on its effective area. Therefore, it is reasonable to conclude that the increased sensitivity of the Au/NPSi electrode is primarily attributable to the increased amount of enzyme immobilized on the sensing electrode's surface. The self-assembly process used in this paper has little effect on the enzyme activity of the Urs, and the sensor shows high sensitivity compared with that using conventional covalent attachment onto planar electrode. Thus, NPSi-based urea sensors offer promising possibilities for the use of a NPSi layer as biosensor element.

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