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ZnO/Cu Nanocomposite: A Platform for Direct Electrochemistry of Enzymes and Biosensing Applications

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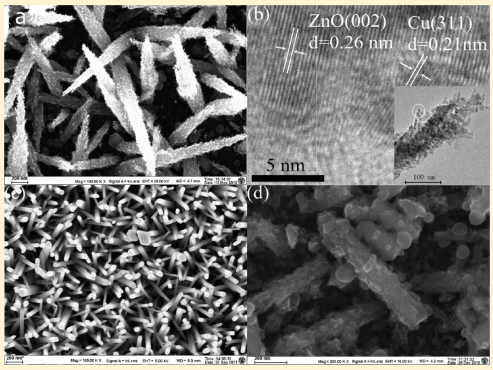
ABSTRACT: Unique structured nanomaterials can facilitate the direct electron transfer between redox proteins and the electrodes. Here, in situ directed growth on an electrode of a ZnO/Cu nanocomposite was prepared by a simple corrosion approach, which enables robust mechanical adhesion and electrical contact between the nanostructured ZnO and the electrodes. This is great help to realize the direct electron transfer between the electrode surface and the redox protein. SEM images demonstrate that the morphology of the ZnO/Cu nanocomposite has a large specific surface area, which is favorable to immobilize the biomolecules and construct biosensors. Using glucose oxidase (GOx) as a model, this ZnO/Cu nanocomposite is employed for immobilization of GOx and the construction of the glucose biosensor. Direct electron transfer of GOx is achieved at ZnO/Cu nanocomposite with a high heterogeneous electron transfer rate constant of 0.67 ± 0.06 s^{-1}. Such ZnO/Cu nanocomposite provides a good matrix for direct electrochemistry of enzymes and mediator-free enzymatic biosensors.



INTRODUCTION

Recently, direct electrochemical biosensors based on the direct electron transfer (DET) between redox proteins and the electrodes have been developed increasingly in the fields of medicine, biotechnology, environmental monitoring, and so on.[1−3] However, DET is difficult for most redox proteins on conventional electrodes due to the deeply immersed redox center of proteins and passivation of proteins after adsorption on electrode surface.[4] Fortunately, unique structured nanomaterials can facilitate DET due to their small size and novel physical and chemical properties for electron-mediating the redox reaction.[5,6]

Nanostructured ZnO has been used to fabricate biosensors owing to its biocompatibility, low toxicity, high electron mobility, and easy fabrication.[7,8] Moreover, ZnO, having a high isoelectric point (pI ∼ 9.5) and positive surface charge for ZnO quantum dots, is beneficial for the adsorption of proteins or enzymes with low pI (e.g., glucose oxidase, GOx, pI = 4.2−4.5) at physiological pH of 7.4 by electrostatic attraction.[9] To date, various ZnO nanostructures, such as nanorods,[10,11] nanosheet,[12] nanobelts,[13] nanoporous,[14] nanodisks,[15] nanoparticles,[16] and radial nanowire array[17] have been prepared and further used to immobilize redox proteins for direct electron transfer. To the best of our knowledge, the traditional approaches to synthesize these ZnO nanostructures are usually by means of a hydrothermal reaction with the assistance of organic reagents.[10−14] However, these methods are maybe not the best for biosensors due to the possible toxicity of organic solvents. Moreover, the conventional ZnO-modified electrode substrates are almost always fabricated by postpasting of ZnO,[12,18,19] which does not enable robust mechanical adhesion and electrical contact between the nanostructured ZnO and the substrate. It is desirable to improve the electrical contaction and sensing performance of ZnO nanostructured electrodes in situ synthesized without using any organic reagent. Here, the desired electrode was fabricated simply by growing prickly ZnO/Cu nanocomposite directly on electrode substrates via a corrosion method and without using any organic reagent. Then a biosensor could be easily constructed by immobilizing the enzymes on the as-prepared electrode.

EXPERIMENTAL SECTION

Reagents and Materials. Indium tin oxide (ITO) sheets (resistance 10 Ω sqr^{-1}) were purchased from Shenzhen YH Co. Chitosan (CS) with 98% deacetylation and an average molecular weight of ∼1 × 10^4 g mol^{-1} was obtained from Yuhuan biomedical Corp. Concanavalin A (Con A), GOx (activity 250 units/mg), horseradish peroxidase (HRP, 250 units/mg), and other reagents, proteins, and solvents were purchased from Sigma and were used without further purification. All other stock solutions were prepared using deionized water.

Fabrication of Prickly ZnO/Cu/ITO Electrode. Two metallic targets of copper and zinc (99.99% pure, 2 in. diameter, and 5 mm thick) were used for depositing Cu−Zn alloy layer, by reactive RF magnetron sputtering, onto ITO substrates (2 × 1 cm^2). The targets were arranged confocally for cosputtering and the substrate to target distance was kept constant at 50 mm with a constant copper power of 100 W and zinc power 40 W. The substrates were cleaned by rinsing in ultrasonic baths of 25% $NH_3·H_2O$−ethanol (1:3, v/v). The total

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pressure was kept constant at 5.0×10^{-5} Pa with Ar and N_2 constant gas flow rates of 10−50 sccm. The deposition time and gas pressure were kept constant at 5 min and 2 Pa, respectively, for all depositions. Before starting the actual experiment, the target was presputtered for 15 min with a shutter located in between the target and the substrate. After that, the Cu−Zn-coated ITO was put into Teflon-lined stainless steel autoclave contained 50 mL of deionized water. After reaction at 90 °C for 18 h, the prickly ZnO/Cu nanocomposite was in situ grown on ITO substrate, which was then rinsed with deionized water and kept in the refrigerator at 4 °C for further use. The in situ synthesized ZnO nanorod on ITO electrode is prepared by the conventional hydrothermal method elsewhere.[20]

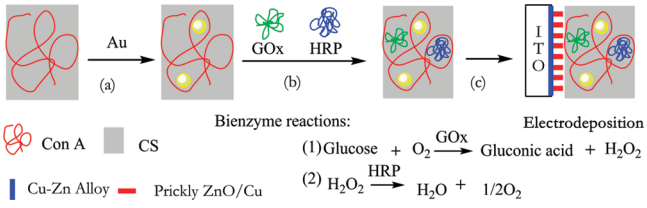
Construction of the Biosensor. For immobilization of the GOx and HRP,[21,22] a homogeneous mixture containing CS, Au nanoparticles (Au), Con A, HRP, and GOx were first prepared as electrodeposition solution for preparation of biocomposite film (BFM). CS powders were dissolved in diluted HCl solution, and a solution with pH near 3.0 was obtained. After undissolved material was filtered, the pH was adjusted to 5.0 using 0.3 M NaOH. Con A stock solution was prepared by 0.1 M PBS (pH 7.4) containing 1.0 mM $CaCl_2$ and 1.0 mM $MnCl_2$. Typically, a homogeneous stock solution of Con A/CS and Au (0.01 mM) solution was formed by blending CS solutions (0.5 wt %, pH 5.5) with Con A (3 mg/mL) and Au and sonicated for 3 min (Scheme 1a). CS-Au/Con A/HRP-GOx solution was obtained by blending such CS-Au/Con A solution with 1.0 mg/mL HRP and 1.0 mg/mL GOx solution and then sonicating for 2 min (Scheme 1b).

For electrodeposition, prickly ZnO/Cu nanocomposite was immersed in CS-Au/Con A/HRP- GOx biocomposite solution and used as the cathode by applying a voltage of −0.1 V for 5 min (Scheme 1c).[21,22] The obtained electrode (CS-Au/Con A/HRP-GOx) was washed with 0.1 M PBS, dried at room temperature for about 5 min, and then stocked at 4 °C in the dry state for 12 h before use.

Apparatus. The electrochemical properties were examined with a CHI 830 electrochemical workstation (Shanghai, China) utilizing a conventional three-electrode system, which consists of a Pt wire as the counter electrode, a saturated calomel electrode (SCE) as the reference, and the prickly ZnO/Cu/ITO as the working electrode. The electrolyte was 0.1 M pH 7.4 PBS solution. EIS measurements were performed in the presence of 5 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1:1) mixture as a redox probe at the formal potential, E_0 = 210 mV, using alternating voltage of 10 mV in the frequency range from 10^5 to 10^{-1} Hz. All experiments were performed at room temperature (25 ± 1 °C).

The prickly ZnO/Cu nanocomposite was characterized using powder X-ray diffraction (XRD, Cu Kα, Bruker D-8 Avance), field emission scanning electron microscopy with energy-dispersive X-ray analysis (FESEM, EDX, JSM-6700F), X-ray photoelectron spectroscopy (XPS, ESCA LAB 220-XL), and high-resolution transmission electron microscopy (HRTEM).

Scheme 1. Schematic of Integrated Glucose Sensor with Prickly ZnO/Cu Nanocomposite[21]



RESULTS AND DISCUSSION

The formation of ZnO nanostructures on the ITO electrode was observed by FESEM (Figure 1a). It was observed that ZnO nanostructures had a diameter range of 30−100 nm and a length of a few micrometers with a prickly shape. Apparently, the nanostructures could provide a high specific surface area to load large amounts of enzymes for high concentration of enzymatic reaction centers. A typical TEM image of the ZnO

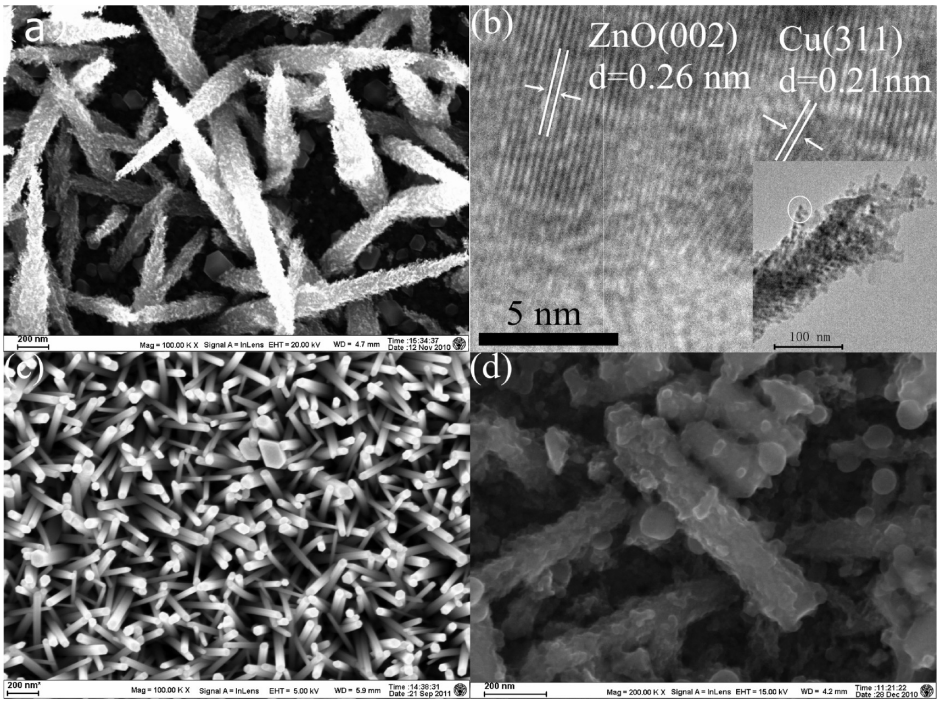


Figure 1. (a) FESEM image of the prickly ZnO/Cu. (b) HRTEM and TEM (inset) images of the nanocomposite. (c) FESEM image of the ZnO nanorods prepared by the conventional hydrothermal method elsewhere. (d) FESEM image of prickly ZnO/Cu after absorption of the CS-Au/Con A/HRP-GOx.

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nanostructure is present in Figure 1b. The TEM image clearly shows prickly ZnO. Some Cu clusters are 2−3 nm in size on the surface of zinc oxide. The presence of Cu cluster was confirmed by examining a HRTEM image (Figure 1b). The high-resolution image demonstrates the interplanar spacing of 0.21 and 0.25 nm, which correspond to the *d*-spacing of Cu(111) and ZnO(002) planes (JCPDS No. 38-1240), respectively.

To invested the growth mechanism of the prickly ZnO, EDX analysis was first used to study the components of the prickly ZnO on the ITO substrate (Figure 2a). It reveals only Zn, Cu,

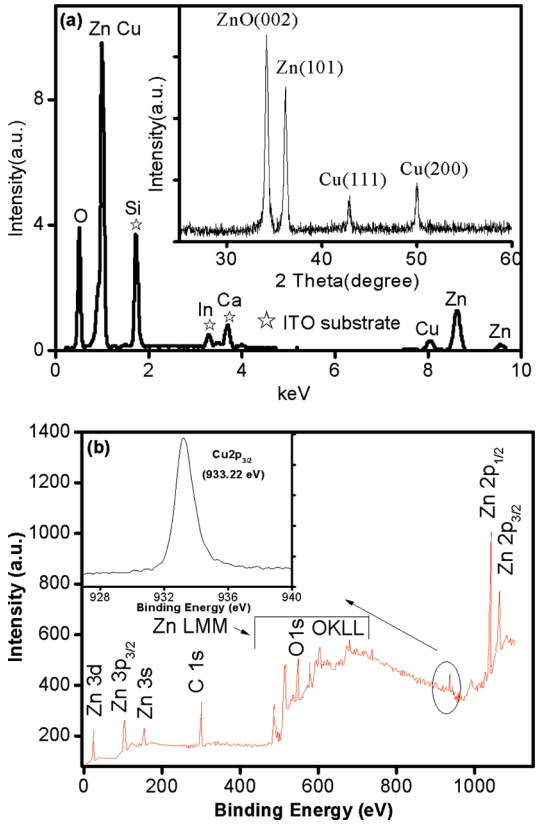

Figure 2. (a) EDX of the ZnO/Cu nanocomposite (inset, XRD of prickly ZnO/Cu nanocomposite). (b) XPS spectrum of the ZnO/Cu nanocomposite (inset, Cu 2p level spectrum).

Si, Ca, In, and O as constituting elements (the Si, Ca, and In come from the ITO substrate). Further, the XRD pattern is shown in Figure 2a. The broad peak is derived from ZnO (JCPDS No. 36-1451) and several small peaks correspond to Cu (matched with JCPDS04-0836), which was consistent with the analysis of HRTEM results above. The XPS spectrum of ZnO was measured to analyze the valence state of Cu in prickly ZnO. All indexed peaks could be ascribed to C, Cu, O, and Zn (Figure 2b). As seen in the inset of Figure 2b, there were two components in the spectrum for Cu 2p core level. The Cu 2p3/2 signals locate around 933.2 eV, which can be attributed to the presence of Cu/ZnO (inset image of Figure 2b).[23]

Considering the above measurements, the crystal growth may be dominated mainly by the corrosion mechanism.[24,25] In our experiment, we found that there was no ZnO nanostructures on the surface of ITO substrate when the Zn-coated substrate was immersed in the same solution for a rather long time. The result is the same as that of the Zn/Cu-coated substrate when performed under nitrogen. However, when the Zn/Cu-coated substrate was immersed into the air-saturated solution, even for 4 h, the surface over the substrate changed to a gray color, and the morphology was altered (Figure 1a). These experiments indicate that electrochemical corrosion occurred. In other words, the mechanism of prickly ZnO/Cu nanocomposite obeys an electrochemical corrosion mode via an oxygen consumption type of corrosion. The corrosion mechanism could be described as $Zn - 2e^- \rightarrow Zn^{2+}$ and $O_2 + 2H_2O + 4e^- \rightarrow 4OH^-$ for anode and cathode, respectively.[24] With further reaction, Zn^{2+} and OH^- combined to form $[Zn(OH)_4]^{2-}$, and then hydroxide precipitates. The precipitates were dehydrated, and ZnO colloids were obtained. Consequently, the prickly ZnO/Cu nanocomposite is formed, which has robust mechanical adhesion and electrical contact of every prickly ZnO/Cu nanocomposite to the ITO electrode. As compared to the ZnO nanorod in situ synthesized by the conventional hydrothermal method (Figure 1c), such type of ITO electrode containing the copper composite has a good electrical conductivity (Figure 3, part b vs c),[26] which is more desirable for constructing the biosensor.

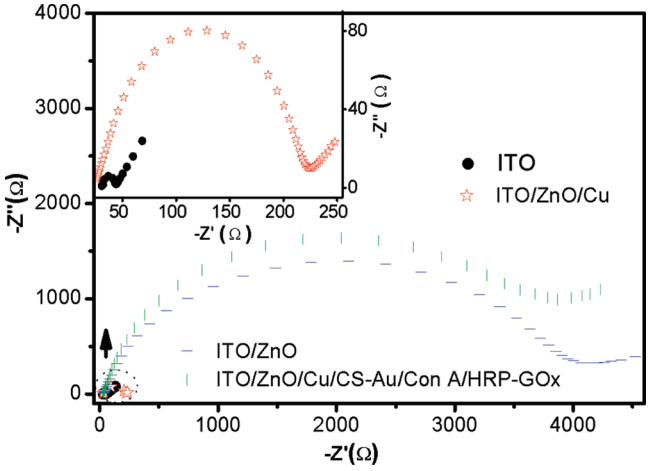

Figure 3. EIS for bare ITO, ZnO/ITO, ZnO/Cu/ITO, and CS-Au/HRP-GOx/Con A/ZnO/Cu/ITO in a solution of 0.1 M KCl containing 5.0 mM $Fe(CN)_6^{3-/4-}$.

The glucose detection is of great importance for diagnosis and therapy of diabetes.[19,27] The prickly ZnO/Cu nanocomposite electrode was employed to immobilize of composite bienzyme for constructing glucose biosensor (Scheme 1). The biocomposite films consists of cross-linked CS matrix and the following functional components: the enzyme GOx to catalyze glucose oxidation generating detecting signal;[28] the HRP to quench undesired H_2O_2 produced by glucose oxidation and increase GOx half-life; Con A to possess biospecific affinity with glycoconjugates such as glycoproteins HRP, GOx[28,29] and polysaccharide CS;[28,30] and multifunctional Au nanoparticles to reinforce the mechanical strength of the membrane and accelerate electron exchange between electrode and proteins.[31] The electrodeposition was performed at reducing potentials, and H^+ in the solution was reduced to H2 at the cathode. Using the locally generated H^+ gradient, acidic side chains of CS were titrated, leading to a change in CS solubility and hence to the controlled deposition of CS film.[28,32] Moreover, special biocomposites could be easily achieved through effective incorporation of functional substrates in the process.[33]

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Therefore, CS-based biocomposites film containing Au/Con A/HRP-GOx was easily immobilized onto the prickly ZnO nanostructure through simple and controllable electrodeposition. The surface morphology of the formed biocomposite film was investigated using SEM (Figure 1d). In comparison to those in Figure 1a, the prickly ZnO in Figure 1d shows larger diameters due to the immobilization of enzymes. It demonstrated that the CS-Au/Con A/HRP-GOx biocomposite film absorbed firmly on the electrode by one-step electrodeposition. Such morphological structure is beneficial to electron transfer between enzymes and electrode. Electrochemical impedance spectroscopy (EIS) was also used to monitor the fabricating process. The redox couple $Fe(CN)_6^{3-/4-}$ is widely used as an electrochemical probe in the electrochemical impedance study, especially for the characterization of biomaterial-modified electrodes.[34,35] Figure 3 showed the Nyquist plots of EIS of the modified ITO. The semicircle diameter of EIS corresponded to the electrotransfer resistance (R_{et}), which controls the electron transfer kinetics of the redox probe at the electrode interface. The bare ITO exhibits an almost straight line, which implies the characteristic of a diffusion-limiting step in the electrochemical process. The R_{et} at the ZnO/Cu/ITO electrode has a small increase (120 Ω), much smaller than that at the ZnO/ITO electrode (1247 Ω), indicating its much improved electroactivity. Upon further immobilization of composite bienzyme, the values of R_{et} increased to 1646 Ω, which is due to the generation of the insulating protein layer on the assembled surface.

Figure 4a shows the typical cyclic voltammogram (CV) responses of CS-Au/Con A/HRP-GOx/ZnO/Cu electrode. As shown in Figure 4a, a pair of well-defined redox peaks, which can be ascribed to the electron transfer between the enzyme and the underlying electrode, is observed at the prickly ZnO/Cu nanocomposite electrode. The anodic peak potential (E_{pa}) and cathodic peak potential (E_{pc}) are located at −0.32 and −0.39 V, respectively. In contrast, no peak was observed at both ZnO/Cu and CS-Au/Con A/ZnO/Cu electrodes. Meanwhile, also no peaks are detected for CS-Au/Con A/HRP-GOx/ZnO electrode. The above results demonstrate that the prickly ZnO/Cu nanocomposite is suitable for the direct electron transfer between enzyme and electrode. Figure 4b shows the effect of scanning rate on the electrochemical response of enzyme on prickly ZnO/Cu nanocomposite electrode. The redox peak current increases as the scanning rate increases. The plotted relationship demonstrates that response current is linearly proportional to the scan rate ranging from 0.1 to 0.35 V s^{-1}, as shown the inset I in Figure 4b. The linear regression equations are $I_{pa}(\mu A) = -0.058\nu$ (mV/s) −1.56, $R^2 = 0.994$; $I_{pc}(\mu A) = 0.062\nu$ (mV/s) − 0.408, $R^2 = 0.996$, respectively. This suggests that the electron transfer process for CS-Au/Con A/HRP-GOx/ZnO/Cu electrode is a surface-confined mechanism in the potential scope. Inset II in Figure 4b shows the relationship between the peak potential E_p and the natural logarithm of scanning rate ln ν for CS-Au/Con A/HRP-GOx/ZnO/Cu electrode in 0.1 M PBS (pH 7.4). Theoretically, the relationship can be described with the Laviron equation[36]

$$E_p = E^{0'} + \frac{RT}{\alpha nF} - \frac{RT}{\alpha nF} \ln \nu$$

where α is the cathodic electron transfer coefficient, n is the number of electrons, and R, T, and F have their usual meanings (R = 8.314 J/mol/K, T = 298 K, F = 96 493 C/mol). On the basis of the data obtained from inset II in Figure 4b, it is concluded that n = 1.9 and α = 2.6. Therefore, the redox reaction between enzyme and ITO was a two-electron transfer process. In order to calculate the value of the apparent heterogeneous electron transfer rate constant (K_s), the following Laviron equation was used[36]

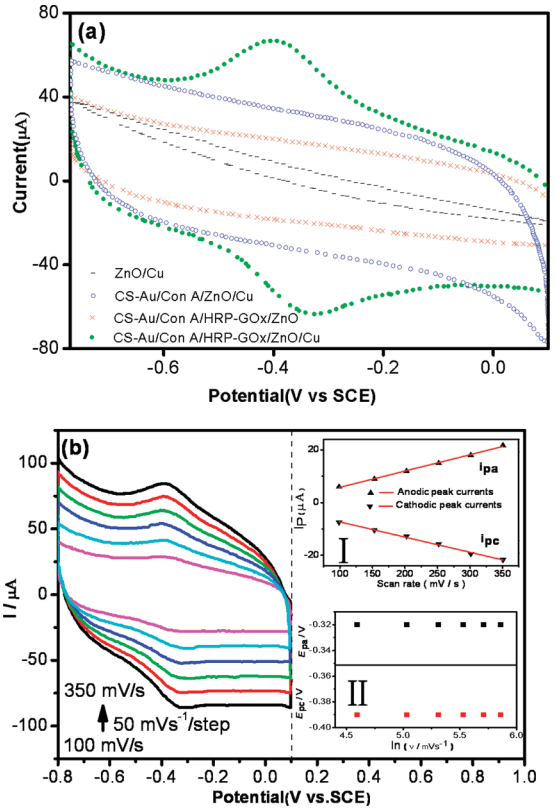

Figure 4. (a) CV curves of different electrodes in N_2-saturated PBS solutions at 0.1 V/s: ITO, CS/Au-HRP-GOx/Con A/ZnO, CS/Au/Con A/ZnO/Cu and CS/Au-HRP-GOx/Con A/ZnO/Cu. (b) CV curves of sensor 1 in 0.1 M PBS (pH 7.4) at different scanning rates. (Inset I) The plot of anodic and cathodic peak currents vs scanning rates. (Inset II) The relation between the peak potentials and logarithm of ν.

$$\log K_s = \alpha \log(1-\alpha) + (1-\alpha)\log(1-\alpha) - \log(RT/nF\nu) - \alpha(1-\alpha) - \log(nF\Delta E_p/2.3RT)$$

Taking a charge transfer coefficient α of 2.6 and a scan rate of 100 mV/s, K_s of enzyme in the CS/Au-HRP-GOX/Con A/ITO is estimated as 0.67 ± 0.06 s^{-1}. This value is close to that obtained at carbon nanotubes (CNT)-HRP/GOx-Nafion (0.55 s^{-1})[37] and higher than that obtained at GOx/HRP-Ag/CNT/CS (1.76 ± 0.08).[38] It is believed that after the electron transfer from enzyme to ZnO, prickly ZnO/Cu nanocomposite provide direct electron pathways for transferring electrons immediately from prickly ZnO/Cu nanocomposite to the ITO electrode. By using the electrochemical version of the Lineweaver−Burk equation,[39] $1/I_m = 1/I_{max} + K_M^{app}/CI_{max}$, $1/I_m$ vs $1/C$ is plotted in inset I of Figure 4b, where I_m is the steady-state current, I_{max} is the maximum current, and C is the glucose concentration. The K_M^{app} value of CS/Au-HRP-GOX/Con A is calculated as 1.47 mM from the intercept and slope of the linear plot, which is much smaller than that of 7.3 mM for the HRP-GOx layered electrode.[40] The smaller K_M^{app} means that the enzyme electrode

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possesses higher enzymatic activity to glucose oxidation and higher affinity toward the substrate.

In air-saturated PBS, dissolved oxygen could participate in the redox process,[19,41] as seen in Figure 5a. The composite film

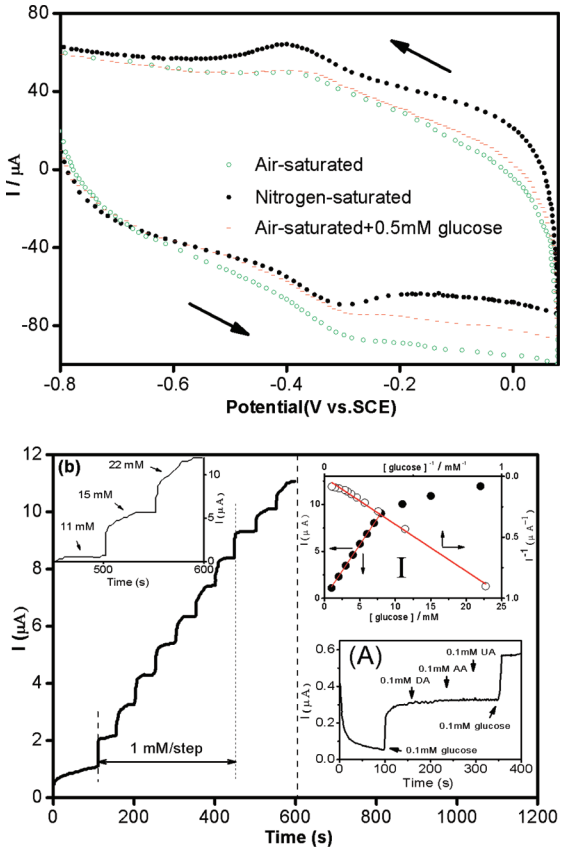

Figure 5. (a) CVs of the CS-Au/Con A/HRP-GOx/ZnO/Cu electrode in N_2- and air-saturated PBS solutions with and without the addition of 0.5 mM glucose. Scan rate: 100 mV s^{-1}. (b) Amperometric response of the CS-Au/Con A/HRP-GOx/ZnO/Cu biosensor to different concentrations of glucose in PBS (0.1 M, pH 7.4) at an applied potential of −0.39 V. (Inset I) The calibration curve (solid square) of the biosensor and the Lineweaver−Burk plot (open circle). The straight line is the linear fit to the calibration curve. (Inset A) Amperometric response of the sensor toward the glucose, dopamine (DA), uric acid (UA), and ascorbic acid (AA) at −0.39 V (vs SCE) in pH 7.4.

modified electrode exhibits an excellent electrocatalytic activity toward the reduction of oxygen, and the anodic peak currents decrease with increasing the exposure time of the anaerobic solution (solid circle) to air (open circle). Once immobilized, GOx retains its biochemical properties toward glucose in aerobic conditions. Moreover, with the addition of glucose to air-saturated PBS, the reduction current decreases (dashed curves). The result further supports that the redox peak of Figure 4b is the result of the direct electron transfer of native GOx.[42] Next, a chronoamperometric experiment at a constant potential of −0.39 V was carried out under constant stirring. Upon the addition of glucose, the monitored current was dramatically increased, as shown in Figure 5b. The 95% of the steady-state current was obtained within 6 s. The inset I in Figure 5 displays the calibration curves of the amperometric response of the biosensor to the concentration of glucose, which presents a linear range from 1 to 15 mM with a correlation coefficient of 0.993. The sensitivity of the biosensor is estimated as high as 97 nA/mM, which is better than many previous reports[38,43,44] and even higher than that of the biosensor constructed with Ag/CNTs (135.9 μA/mM).[39] The detection limit is further estimated as ∼0.04 mM at a signal-to-noise ratio of 3. This value is close to that obtained for nitrogen-doped carbon nanotubes.[45,46]

At the applied potential of −0.39 V, two main interferents were investigated by using the relevant physiological levels (∼0.1 mM) of these common interfering species: 0.1 mM ascorbic acid (AA), uric acid (UA), and dopamine (DA). As compared to the 0.1 mM glucose, no appreciable signal was observed for the successive injections of interferents into the electrolyte solution (inset A of Figure 5b). When the biosensor was stored at 4 °C and measured at intervals of 5 days, it retained about 95% of its original sensitivity after 15 days. The fabrication reproducibility of five electrodes, made independently, showed reproducibility with the cumulative variation of 2.1% for the detection of glucose.

CONCLUSIONS

In summary, we developed a facile method for preparing the prickly ZnO/Cu nanocomposite. The defining feature of this method is its in situ synthesis, without using any organic reagent. The results showed that the mechanism of prickly ZnO/Cu nanocomposite obeys an electrochemical corrosion mode. Such prickly ZnO/Cu nanocomposite with a high-aspect ratio is favorable to immobilize the biomolecules and construct biosensors. Comparative studies revealed that the nanostructure of the prickly ZnO/Cu nanocomposite offers significant advantages over ZnO nanorod in facilitating direct electron transfer. Direct electron transfer of GOx is achieved for prickly ZnO/Cu nanocomposite with a high heterogeneous electron transfer rate constant of 0.67 ± 0.06 s^{-1}. The prepared reagentless mediator-free third-generation biosensor displayed good sensitivity, wide linear range, low detection limit, and fast response for the detection of glucose. The prickly ZnO/Cu nanocomposite proved to be a promising matrix for direct electrochemistry of proteins and biosensors.

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Notes

The authors declare no competing financial interest.

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