Preparation of the glucose sensor based on three-dimensional ordered macroporous gold film and room temperature ionic liquid

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oxidase (GOD). As a result, FAD becomes closer to the electrode surface. Thereby, the bioelectrocatalytic activity of GOD is enhanced, and the sensitivity of glucose biosensors is improved[8]. Tang et al. reported an amperemetric glucose biosensor based on the immobilization of GOD and gold nanorod mixture onto the electrode surface, with the detection limit of 2×10^{-5} M[9]. Lee et al. produced a highly sensitive glucose sensor via layer-by-layer (LBL) assembly of multi-walled carbon nanotubes, GNPs, thiol functionalized polyaniline and GOD, with a linear response range of 1—9 mM[10]. Another new type of dual-enzyme biosensor with the detection limit of glucose down to 0.5 μM[11] was fabricated in our lab, in which GOD and horseradish peroxidase (HRP) were simultaneously immobilized in the GNPs-mesoporous silica SBA-15 composite. Constructions of these highly sensitive glucose sensors have great promotion on the development of non-invasive glucose monitor.

Three-dimensional ordered macroporous (3DOM) materials modified electrodes can provide large active surface, which are promising to increase functional density and facilitate electron exchange. Xia and co-workers reported the use of a 3DOM gold film modified electrode for the direct electron transfer of hemoglobin[12] and the fabrication of nonenzymatic glucose and methanol sensors[13,14]. 3DOM Prussian blue (PB) film[15] and 3DOM gold film[16] modified electrodes have also been prepared for glucose detection. Li et al. immobilized HRP onto the 3DOM titania modified electrode, and developed a highly sensitive photoelectric sensor for the determination of H_2O_2[17]. In these systems, the film efficiency or sensitivity was significantly higher than that of nonporous films.

Room temperature ionic liquids (ILs) are ionic compounds consisting of organic cations and various anions, which are in liquid state at ambient temperature and belong to nonaqueous polar solvents. They have many unique characteristics such as negligible vapor pressure, relatively high ionic conductivity, good electrochemical stability and good physicochemical stability, so they are referred to as "green" solvents[18]. Especially, because of the wide electrochemical windows and excellent biocompatibility, ILs have been considered as good media for electrochemical reactions. Proteins such as enzymes can also keep their activity and stability in the ILs better than in the conventional organic solvent or aqueous solution[19]. Dong et al. studied the direct electrochem-

istry of microperoxidase and HRP immobilized in [BMIm][PF$_6$]-carbon composite material, finding that the prepared modified electrode could catalyze the reduction of O_2 and H_2O_2[20]. Wang et al. investigated the activity, stability and the catalytic ability of a series of heme proteins fixed in the agarose hydrogel in the presence of hydrophobic [BMIM]PF$_6$ and hydrophilic [BMIM]BF$_4$ separately[21]. By simply incorporating ILs into the carbon paste electrode, the carbon ionic liquid electrode (CILE) exhibited excellent electrochemical characteristics and was applied to the electrochemical biosensors. Sun et al. electrodeposited Co nanoparticles on the surface of CILE, based on which myoglobin (Mb) was immobilized and the direct electrochemistry of Mb was studied. The prepared modified electrode exhibited good electrochemical catalysis to H_2O_2 and trichloroacetic acid[22]. A new type of composite material, gellan gum-[BMIM]BF$_4$, has been prepared by Zheng et al. and used to entrap HRP, with the detection limit of H_2O_2 down to 0.02 μM[23].

Herein, GOD-*N,N*-dimethtylformamide (DMF) and [BMIm][BF$_4$] composites were modified on a 3DOM gold film electrode, thus a novel glucose biosensor was fabricated. The immobilized GOD exhibited a pair of well-defined reversible peaks in pH 7.0 phosphate buffer solutions (PBS), resulting from the redox of FAD in GOD. The experimental results show that IL ([BMIm][BF$_4$]), DMF and 3DOM gold film are crucial for the direct electrochemistry of GOD. It is believed that the large active area of 3DOM gold film can increase the amount of immobilized GOD, and the application of IL enhances the stability of GOD and facilitates the electron transfer between GOD and electrode. The synergetic effect of DMF can help GOD to maintain its bioactivity better. GOD immobilized on the electrode exhibits favorable electrocatalytic property to glucose, with a linear range from 10 to 125 nM and a detection limit of 3.3 nM at a signal-to-noise ratio of 3. The apparent K_m (Michaelis-Menten constant) for the enzymatic reaction is 0.018 mM.

2 Experimental section

2.1 Materials

GOD (EC 1.1.3.4, 35.5 units/mg) was purchased from Sigma-Aldrich. The monodisperse silica sphere with a diameter of 250 nm was obtained from Alfa Asear. [BMIm][BF$_4$](1-butyl-3-methylimidazolium tetrafluoroborate) (pu-

rity > 99%) was purchased from Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences and dried in vacuum at 60℃ for 24 h before use, and stored in a desiccator as well. Glucose was purchased from Sinopharm Chemical Reagent Co., Ltd. Chitosan (Chi) was purchased from Nanjing Debio Co., Ltd. Au-Cl₃·HCl·4H₂O was obtained from Shanghai Sinopharm Chemical Reagent Co., Ltd. (SCRC). Phosphate-buffered saline (PBS buffer, 50 mM) was prepared by varying the ratio of NaH_2PO_4 and Na_2HPO_4. The 5 mg/mL GOD solution was prepared in the pH 7.0 PBS buffer solution, and stored at 4℃. All other chemicals were of analytical grade and were used without further purification. Ultrapure fresh water was obtained from a Millipore water purification system (MilliQ, specific resistivity > 18 M : ·cm $^{-1}$, S.A. Molsheim, France) and used in all runs.

2.2 Apparatus

The cyclic voltammetry (CV) and differential pulse voltammetry (DPV) measurements were performed on a CHI660A electrochemical workstation (Shanghai CH Instruments Co.). A conventional three-electrode system was used comprising a platinum foil as the auxiliary electrode, a saturated calomel electrode (SCE) as the reference and the 3DOM gold film modified electrode as the working electrode. All potentials herein are referenced to the SCE. The geometric area of the working electrode was controlled by insulating tape covering the edges of SiO_2 layers and determined to be 0.07 cm².

2.3 Fabrication of the glucose biosensor

The preparation of 3DOM gold film has been described in our previous paper[24]. IL, DMF and 5 mg/mL GOD were mixed together in accordance with the volume ratio of 3:5:9[25] into a homogeneous mixture. 6 ㎕ of the mixture was dropped on the surface of 3DOM gold film electrode and dried overnight at 37℃. Finally, 6 ㎕ of Chi solution (1 mg/mL) was cast on the surface of dried electrode. The fabricated modified electrode was denoted as Chi/IL-DMF-GOD/3DOM and stored at 4℃ when not in use. Prior to each experiment, the modified electrode was immersed in pH 7.0 PBS for 30 min to remove physical absorption. Other modified electrodes such as Chi/GOD/3DOM, Chi/IL-GOD/3DOM, Chi/DMF-GOD/3DOM, Chi/IL-DMF/3DOM and Chi/IL-DMF-GOD/Au were prepared by a similar procedure.

2.4 Electrochemical measurements

All experiments were performed at room temperature in 50 mM pH 7.0 PBS buffer as the supporting electrolyte. The experiments for glucose determinations were carried out in air-saturated solutions, and other experiments were performed in the solutions deoxygenated by bubbling highly pure nitrogen for 30 min and maintained under nitrogen atmosphere during measurements.

3 Results and discussion

3.1 Direct electrochemistry of GOD

Figure 1 shows the CVs of different modified electrodes in deoxygenated PBS solution. Curve a corresponds to Chi/IL-DMF-GOD/3DOM modified electrode, and a pair of obvious redox peaks can be observed with the peak potentials of 0.438 V and 0.396 V, respectively. The peak-to-peak difference was 42 mV and the ratio of peak currents was about 1, indicating a reversibly direct electrochemical response of GOD. Curve d corresponds to Chi/IL-DMF/3DOM modified electrode, and no peak could be observed, indicating that the pair of redox peaks in curve a could be attributed to the direct electrochemical behavior of GOD. Curve e is the CV obtained at Chi/GOD/3DOM modified electrode and a pair of stable reversible redox peaks could also be observed, indicating the direct electrochemical behavior of GOD. However, compared with curve a, the peak potentials shifted negatively and the peak currents decreased significantly, indicating IL could enhance the conductivity and promote the electron exchange between GOD and electrode greatly. The peak currents of curve b which corresponds to Chi/IL-GOD/3DOM modified electrode were very low, and the peaks would disappear after four times of continuous scanning. A low redox response of GOD is shown in curve c, corresponding to Chi/DMF-GOD/3DOM modified electrode, and the reduction current would reduce nearly 50% after five times of continuous scanning. In addition, we also studied the direct electrochemical behavior of GOD at a conventional planar gold electrode (Au) and no peaks could be observed (curve f). These results show that 3DOM gold film structure, as well as the synergetic interaction of IL and DMF with GOD could both promote the direct electrochemical behavior of GOD. The 3DOM gold film was composed of gold nanoparticles, which could provide an environment similar to that of GOD in native

system and gave the protein molecules more freedom in orientation, thus reducing the insulating property of the protein shell for the direct electron transfer and facilitating the electron transfer through the conducting tunnels of colloidal gold[26]. A hydrogen bond formed between GOD and DMF, strengthened the hydrophobicity of the microenvironment, and formed a cavity for the entrapment of GOD. As a result, the biological activity of GOD has been maintained[21]. The application of IL can not only maintain the biological activity of GOD, but also enhance the electrochemical activity of GOD, promoting the electron exchange. The interactions in the composites based on IL, DMF and GOD are complicated, mainly including electrostatic and hydrogen bond, van der Waals force, hydrophilic and hydrophobic effects[27].
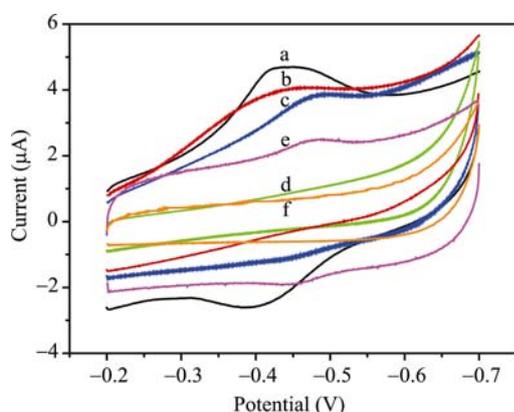

Figure 1 CVs of (a) Chi/IL-DMF-GOD/3DOM, (b) Chi/IL-GOD/3DOM, (c) Chi/DMF-GOD/3DOM, (d) Chi/IL-DMF/3DOM, (e) Chi/GOD/3DOM and (f) Chi/IL-DMF-GOD/Au in nitrogen-saturated 50 mM pH 7.0 PBS at 100 mV·s^{-1}.

3.2 The influence of scan rates on the direct electrochemistry of GOD

FAD, a part of the GOD molecule, is known to undergo redox reaction where two protons and two electrons are released or taken up[28,29]. In GOD, FAD is deeply seated in a cavity and therefore not easily accessible for electron exchange between FAD and the electrode surface. According to the conclusion of Ianniello et al., the electrochemical response of GOD immobilized on the heterogeneous surface is due to the redox of FAD[30].

We have studied the influence of scan rate on the direct electrochemistry of GOD. As shown in Figure 2, along with the increase of scan rates ($20-600$ mV/s), the peak currents increased too, while the peak potentials almost kept unchanged. The redox peak currents

were proportional to the scan rates in the range less than 100 mV/s (inset (a) in Figure 2), indicating a typically surface-controlled reversible process. According to Laviron equation $i_p = nFQv/4RT$[31], where n is the number of electron involved in the electrochemical process, F is Faraday constant, Q is charge integration of reduction peaks, v is scan rate, R is the molar gas constant, and T is thermodynamic temperature, n can be calculated to be 2.18, i.e., two electrons have been involved in the process. In addition, according to Faraday′s law $Q = nFA\Gamma^*$, where A is the actual area of the electrode, and Q is the quantity of charge integrated from the reduction peak of Chi/IL-DMF-GOD/3DOM at scan rate less than 100 mV/s. The surface coverage of GOD, Γ^*, is calculated to be 4.18×10^{-12} mol·cm^{-2}. This shows that the immobilization of GOD on the 3DOM gold film modified electrode is in a monolayer level[32], which might facilitate direct electron transfer between GOD and the electrode surface. When $n'E_p < 200$ mV, the direct-electron-transfer rate constant K_s was analyzed using the Laviron formula $K_s = mnFv/RT$[31]. The plot of cathodic peak potentials vs. the logarithm of scan rates gave a charge transfer coefficient D of 0.52. Then K_s was estimated to be 1.95 s^{-1}, which could be ascribed to the high conductivity of the interconnected 3DOM gold film and the present IL.

When the scan rates were larger than 100 mV/s, the redox peak currents were proportional to the square root of scan rates, $v^{1/2}$ (inset (b) of Figure 2). Considering the ratio near unity for the cathodic to anodic peak currents,
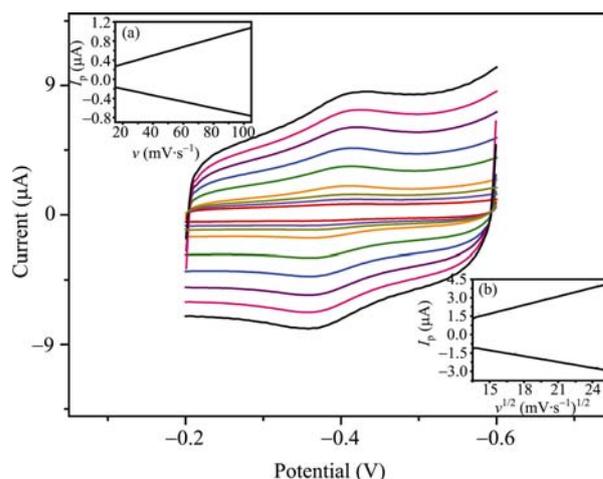

Figure 2 Cyclic voltammograms of Chi/IL-DMF-GOD/3DOM in nitrogen-saturated 50 mM pH 7.0 PBS at 20, 40, 60, 100, 200, 300, 400, 500 and 600 mV·s^{-1} (from inner to outer). Inset (a): Plots of peak currents vs. v. Inset (b): Plots of peak currents vs. $v^{1/2}$.

the electrode reaction was a diffusion-controlled reversible process, illustrating that the diffusion of protons from the solution to the electrode surface was the rate-limited step at the scan rates of more than 100 mV/s.

3.3 The effect of pH on the direct electron transfer of GOD

We have studied the direct electrochemistry of GOD in solutions with different pH values. CV of Chi/IL-DMF-GOD/3DOM showed a strong dependence on solution pH as demonstrated in Figure 3. Within the pH range of 6.0－9.0, there was a pair of reversible and stable redox peaks in CV curve. However, the CV curves were distorted when the pH was less than 6.0 or greater than 9.0, implying denature of GOD. The increase in solution pH caused a negative shift in both cathodic and anodic peak potentials. The plot of the formal potential ($E^{\circ'} = (E_{pa} + E_{pc})/2$) vs. pH produced a line with the slope of 61 mV/pH ($R^2 = 0.997$) (inset of Figure 3), which was close to the expected value of 59 mV/pH, indicating that two protons and two electrons participated in the electron transfer process. The reaction equation was GOD(FAD) + 2e + 2H$^+$ = GOD(FADH$_2$), which was consistent with the reports in the literature[33]. Obviously, the maximum current response and best reversibility occurred at pH 7.0, so all our experiments were carried out in pH 7.0 PBS.

3.4 Determination of glucose in solutions

Upon addition of glucose to air-saturated PBS, the reduction current response of oxygen decreased, as shown
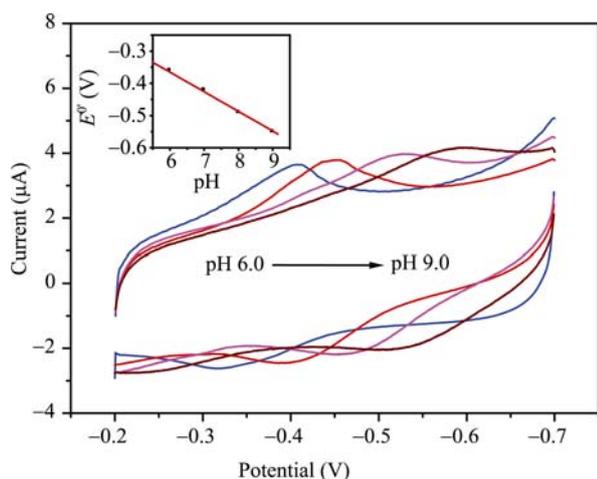
Figure 3 CVs of Chi/IL-DMF-GOD/3DOM in nitrogen-saturated 50 mM PBS with different pH values at 100 mV·s^{-1}. Inset: Plot of formal potential $E^{\circ'}$ vs. pH.

in Figure 4. The peak currents decrease ($'i_p$, compared with the blank solution) increased with an increasing glucose concentration. Glucose is the substrate of GOD, and its presence will result in an enzyme-catalyzed reaction as follows: Glucose + GOD(FAD)→ gluconolactone + GOD(FADH$_2$). The GOD(FADH$_2$) would be oxidized to GOD(FAD) by dissolved oxygen under the air-saturated condition. The addition of glucose restrained the reductive reaction of dissolved oxygen and led to the decrease of reduction current. The inset in Figure 4 shows the plot of $'i_p$ vs. glucose concentration. The linear response of the sensor was from 10 to 125 nM with R^2 of 0.997 and a detection limit of 3.3 nM at a signal-to-noise ratio of 3. The sensitivity of Chi/IL- DMF-GOD/3DOM to glucose was found to be 0.06 ㎀/nM, which is higher than that in previous reports[25,34].
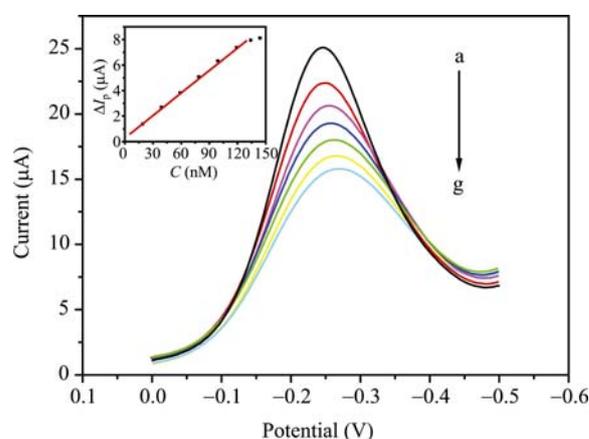
Figure 4 DPVs of Chi/IL-DMF-GOD/3DOM in air-saturated 50 mM pH 7.0 PBS and 0, 20, 40, 60, 80, 100 and 120 nM glucose (from a to g). Inset: plot of $'i_p$ vs. glucose concentration.

According to Lineweaver-Burk equation $1/I_{ss} = 1/I_{max} K_m/(I_{max}C_s)$[35], where I_{ss} is the steady-state current, I_{max} is the intercept on the current axis, K_m is the apparent Michaelis-Menten constant, and C_s is the concentration of substrate, the corresponding plot yielded an "apparent" K_m of 0.018 mM, indicating good biocompatibility of the microenvironment

3.5 Stability and reproducibility of the GOD sensor

The stability of Chi/IL-DMF-GOD/3DOM modified electrode has also been studied. When the enzyme electrode was stored at 4℃, it retained 94% of its initial current response for glucose after intermittent use over a 12-day period. Thus, the presence of 3DOM gold film, IL and DMF provided a suitable microenvironment for

efficient retaining of the enzyme activity of GOD. The current responses of six independent sensors were examined in air-saturated PBS solution containing a glucose concentration of 50 nM, showing an acceptable reproducibility with a relative standard deviation (RSD) of 6.8%. The preparation of this sensor is simple to control with good reproducibility, and thus the batch production can be achieved.

4 Conclusions

A novel type of glucose sensor has been fabricated on the basis of 3DOM gold film modified electrodes. The 3DOM gold film was composed of numerous gold nanoparticles which provided a good microenvironment for the immobilization of protein. The three-dimensional interconnected porous network of 3DOM gold film increased the density of functional groups and provided good conductivity. The large active surface area of 3DOM could promote the adsorption of single-layered protein molecules. The application of IL enhanced the electrochemical activity of GOD and promoted electron transfer. The synergetic interaction of DMF, IL and GOD maintained the bioactivity of enzyme. Chitosan was used to coat the modified electrode, preventing the leakage of GOD. The sensor exhibited favorable electrocatalytic property to glucose, with a linear range from 10 to 125 nM, a detection limit of 3.3 nM, and the sensitivity of 0.06 ₧A/nM. In addition, the sensor presented good stability and reproducibility. The development of this sensor opens a new path for 3DOM gold film and IL in highly sensitive biological detection and sensor miniaturization.

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