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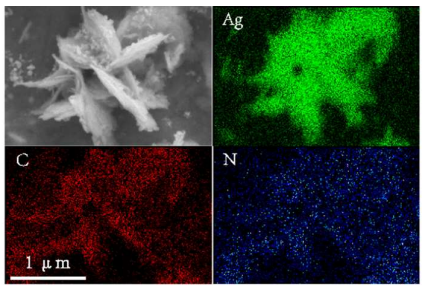

A bio-composite was assembled by in situ reducing Ag^{+} to Ag in glucose oxidase solution and used to detect glucose.

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ARTICLE

In situ growth of metallic sliver on glucose oxidase for highly sensitive glucose sensor

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Qiang-Qiang Sun, Ya-Nan Yu, Jin-Jin Li, Shu-Juan Bao *

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This work presents a new idea to fabricate an enzyme glucose sensor. A bio-composite was assembled by in situ reducing Ag^+ to Ag in the glucose oxidase (GOD) solution. In this way, metallic silver can directly deposit onto GOD surface and induce a tight contact between Ag and GOD. The obtained composites were characterized by FESEM, EDS mapping, FTIR, CD, and electrochemical measurements. The Ag-GOD composite formed by in situ reducing process exhibits facile, direct electrochemistry and good electrocatalytic performance without any electron mediator. The designed glucose biosensor shows high sensitivity, high selectivity, long life and accurate measurement in real serum sample, which is contributed to a fast, direct electron transfer due to close proximity between Ag and GOD.

Introduction

Glucose detection is very important in many types of studies from food processing and fermentation to clinical, biological, and chemical [1-4]. Techniques including fluorescence, colorimetric, flow injection, and electrochemistry analysis have been developed for this purpose [5-8]. Among of these detection techniques, amperometric biosensors, which are based on direct electron transfer (DET) between electrode and glucose oxidase (GOD), have attracted much attention due to their high sensitivity, excellent selectivity, wide test range, and easy operation [9]. Unfortunately, deeply buried redox centers in enzymes and instability of the biological matrix upon interaction with electrode surfaces make direct electrochemistry of most redox enzymes on bare or "naked" solid electrodes very difficult. The key for designing a high performance biosensor is to understand how to efficiently transfer an electron directly between the electrode and enzyme.

In the past few decades, many works on producing such a biosensor have been performed. Nanomaterials like gold, various carbon materials, titanium dioxide, and other conductors have been introduced to initiate direct electron transfer [10-13]. Various morphologies of these materials, including nanowire, nanorods, hollow nanospheres, nanofilm, and nanoparticles were used to enhance the electro-catalytic performance of enzymes, which were restricted due to their small size, high specific area, and appropriate pore size distribution [14-18]. However, since GOD is freely soluble in water, enzymes cannot be immobilized on nanomaterial simply by an adsorption process. Hence, various methods have been developed to effectively immobilize GOD on test electrodes. For example, the cross-linking method of an immobilized enzyme is easy to operate and has good stability. However, this method leads to reduced electrocatalytic activity of the enzyme and long response time. Encapsulation is commonly used to immobilize an enzyme while also improving its stability. This method is easy to control and can be used repeatedly, but the enzyme has a long response. The enzyme immobilized by covalent-bond combination method has a quick response and nice stability; unfortunately, this method weakens the enzyme's activity and has a complex operation. Although some of these nanomaterials and immobilized method could help enzyme-induced, direct electrochemistry reactions, there is still great challenge in developing new ways for better activity, specificity, and stability for super bioelectrocatalysis.

Here, we report on a new, simple in situ growth method to realize effective immobilization of GOD. In our work, silver ions are reduced to metallic silver and in situ deposited on the surface of GOD to form Ag-GOD composite. For comparison, the Ag and GOD composite was also prepared by the conventional direct adsorption method. The results indicate that the sensor prepared based on the former composite has a higher sensitivity, quicker response, and better stability. More importantly, this approach reported here is a new method to immobilize bio-enzyme for sensing. The nanomaterial which could be reduced not only silver but other metals on the bio-enzyme. It should be noted that the reaction condition must be moderate enough to ensure enzymes active.

Experimental

Reagents

The glucose oxidase, ascorbic acid (AA), dopamine (DA) and uric acid (UA) were purchased from Sigma Chemical Co. Fetal bovine serum as a real sample is obtained from Gibco, Invitrogen, USA. $AgNO_3$ is from Fluka Co., D-maltose and D-glucose were purchased from KeLong Chemical Reagent Co., (Chengdu, China). Ammonium hydroxide purchased from ChuanDong Chemical Reagent Co., (Chongqing, China). They were used as received, without further purification. 0.1 M phosphate buffer solutions (PBS) consisted of Na_2HPO_4 and NaH_2PO_4, were employed as supporting electrolyte. The

desired solution pH (7.4) was adjusted by different amount of 0.2 M Na_2HPO_4 and NaH_2PO_4 solutions. Deionized water was used throughout.

The preparation of the materials

Initially, 0.01 M $AgNO_3$ and maltose solution were prepared followed by dissolving 2 mg GOD into 100 μL maltose solution containing 3 μL 3% ammonium hydroxide. After mixing uniformly, 10 μL of prepared $AgNO_3$ was added into the miscible liquids above. After that, the mixture was shaken a few minutes and put in 4 °C refrigerator for 24 h. This sample is named as Ag-GOD. For comparison, metallic silver was first synthesized by the same method but without GOD at room temperature. Next, the prepared silver was mixed with GOD and stored in 4 °C refrigerator for another 24 h. The mixture is abbreviated as Ag/GOD. Ag-GOD composites with different amount of Ag were synthesized by change the amount of $AgNO_3$.

Characterization of the materials

The morphologies of bioconjugates were characterized by FESEM (S-4800, Japan). The element distribution is measured by EDS Mapping. FTIR was used for analyzing the change of functional groups. The Circular dichroism (CD) spectroscopy was taken using a biologic MOS500 spectropolarimeter. Quartz cells of 0.5mm optical path length were used for all CD measurements of GOD (GOD concentration =1mg/mL) and Ag-GOD solutions. Deionized water was used to prepare these solutions.

Fabrication of working electrode

Glass carbon electrodes (GCEs) with a diameter of 3 mm were polished with 0.3 and 0.05 μm alumina powder, followed by thorough rinsing with deionized water. The electrodes were then dried at room temperature, and 5 μL bioconjugates were dropped onto the center of GCEs. The latter was left to dry at room temperature, after which 5 μL of 2.5% Nafion was subsequently placed on the electrode surface to form a Nafion membrane, which was used to fix the GOD-impregnated Ag materials. The electrode fabricated with Ag-GOD is expressed as Ag-GOD/Nafion, while the electrode made by sample Ag/GOD is marked as Ag/GOD/Nafion.

Results and discussion

The surface morphologies of the Ag and GOD composites obtained by different method have been investigated by FESEM. It is clear that they have different morphologies as shown in Fig. 1. Samples of Ag-GOD (A) contain some aggregations of nanoplates and nanoparticles. The diameter of the aggregations is about 1 μm. Ag/GOD composites (B) consist of nanoparticles and some flocs. The diameter of the nanoparticles is far smaller than that of Ag-GOD, around 100 nm. There are several reasons why the differences between these two samples are so distinctive. It is well known that GOD is a dimeric protein, which is covered with carbohydrate chains[19]. In the reduction process, positive silver ions can first be adsorbed onto GOD molecules and then are in situ reduced by maltose. Thus, Ag deposits directly on the surface of GOD and tightly bond with GOD to form Ag-GOD aggregations, in which Ag particles can contact with GOD closely. For Ag/GOD, GOD is simply mixed with Ag nanoparticles.

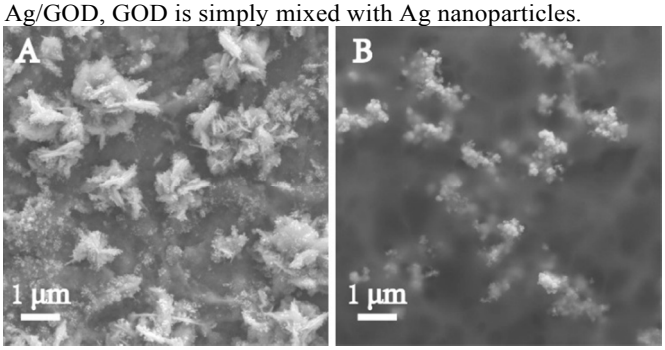

Fig. 1. FSEM images of bio-composites obtained by in situ growth (A) and conventional direct adsorption method (B).

In order to prove silver has in situ grown onto GOD successfully in the synthesis process, Ag, C, and N element distributions have been investigated by EDS mapping. As seen in Fig. 2, Ag-GOD samples (left part) have a similar graphic pattern just like the electronic image; that is to say, Ag, C and N have a same distribution. Because GOD contains C and N elements, we can infer that Ag has successful in situ growth on the surface of GOD. While only Ag distribution is similar with the electronic image in the right side, we can conclude that GOD does not adsorb on silver well in the conventional direct adsorption process.

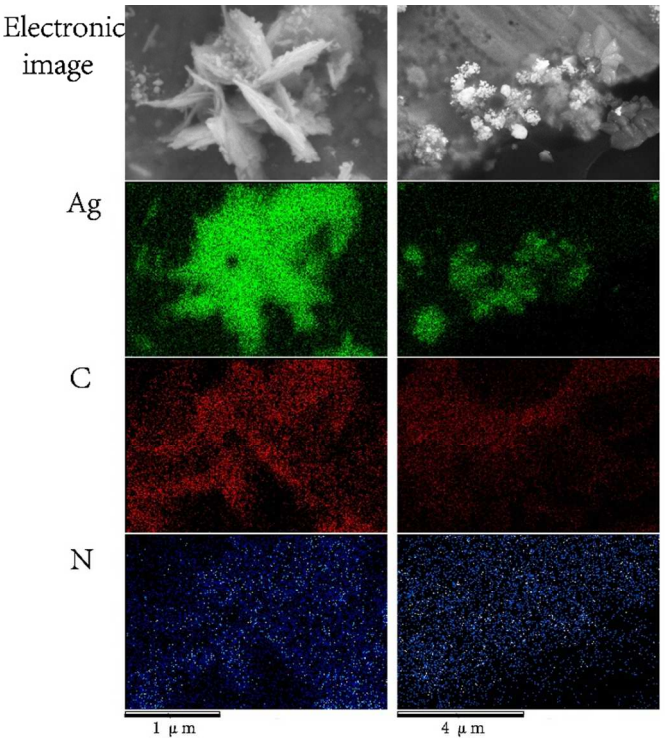

Fig.2. Electronic images and elements distributions of the obtained sample. (Ag-GOD (left) and Ag/GOD (right)).

The in situ growth method can ensure that the enzyme and material are combined closely. FTIR and electrochemical measurements are further performed to estimate the effectiveness of GOD immobilization in different methods. In Fig. 3A, two IR absorption bands from a are centered at 1650

and 1550 cm^{-1} that are ascribed to the typical amide I and amide II adsorption bands of GOD, respectively [20]. In sample Ag/GOD (c), the relative intensity of IR absorption of amide I and amide II band remains constant, while in the sample (b), a slight red shift with respect to the amide peak positions of GOD is observed. This shift may come from the force change among amines, OH groups and silver, which caused by electrostatic interaction in the reaction process due to their different electrical property. Further red shift also means the system has lower energy [21], which indicate that the combination of Ag and GOD in situ reaction is more stable than that of simple adsorption process. CD spectroscopy was further used to characterize the fresh GOD and Ag-GOD colloid. The CD spectrum in Far-UV region (200-250 nm) is usually used to determine the secondary structure change of protein. As shown in Fig.3B, the CD spectra exhibited two negative bands at 208 and 222 nm, which is representative of the α-helix structure of protein. The band at 208 nm corresponds to $\pi \rightarrow \pi^*$ transition of the α-helix, whereas the band at 222 nm corresponds to $\pi \rightarrow \pi^*$ transition for both the α-helix and random coil. The results displayed in Fig. 3B indicated that the structure of native GOD predominately contain α-helix conformation [22]. From 200 to 250 nm, the CD spectra of the fresh GOD and Ag-GOD colloids are almost similar, but he peak has slightly changed at about 205 nm. It indicated that the GOD have been adsorbed on the surface of silver nanoparticles [23]. Only when the state of GOD have a little transformed this change can happen. And the small transformation should be caused by the combination with Ag nanoparticle. Actually, the similar secondary structure content of GOD and Ag-GOD suggested that the secondary structure of GOD was preserved in the presence of Ag nanopartilces [23 24].

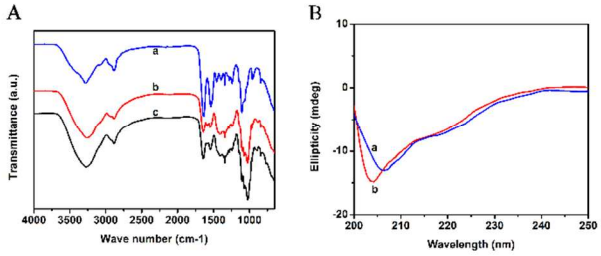

Fig.3. (A) Infrared spectrograms comparison of GOD (a), sample Ag-GOD (b) and sample Ag/GOD (c). (B) Circular dichroism (CD) spectroscopy of Ag-GOD nano-composites (b) and fresh pure GOD (a).

The fabricated electrodes are employed as biosensor for the glucose sensing in order to compare the bioactivity of the GOD immobilized by different ways. The cyclic voltammograms (CVs) of the Ag-GOD electrode (a) and Ag/GOD electrode (b) are shown in Fig. 4A. It is apparent that the Ag-GOD electrode exhibits a pair of obvious symmetric peaks located at about -0.45 V and -0.5 V. This indicates that the electro-catalytic performance of GOD had been improved with help of Ag, while the Ag/GOD electrode displays similar behaviour but has weak redox peaks. It is concluded that direct electron transfer of GOD on the Ag-GOD electrode is more effective than that of the Ag/GOD electrode. In the in situ growth process, silver deposits on the surface of GOD directly and tightly bonds, which is not only beneficial for direct electron transfer but could protect GOD from dissolving into an electrolyte during the test process. Cyclic voltammograms of the Ag-GOD electrode in N_2-saturated (a) and air-saturated (b) PBS are presented in Fig. 4B. It is evident that the oxidation current increases and reduction current decreases in air-saturated PBS compared to the results of N_2-saturated PBS, suggesting that the Ag-GOD fixed on the electrode can electrocatalyze the reduction of dissolved oxygen. The electrode modified with GOD and nanomaterials can electrocatalyze the reduction of dissolved oxygen according to following equations equations [25]. The direct electrochemistry of GOD on the electrode involves a two-proton redox reaction as equation 1:

$$GOD(FAD) + 2e^- + 2H^+ \rightleftharpoons GOD(FADH_2) \qquad 1$$

GOD ($FADH_2$) further electro-catalyze the reduction of dissolved oxygen according to equation 2 as shown in Fig. 3A.

$$GOD(FADH_2) + O_2 \rightleftharpoons GOD(FAD) + H_2O_2 \qquad 2$$

In the presence of glucose, the electrocatalytic reaction is restrained due to the enzyme-catalyzed reaction:

$$Glucose + GOD(FAD) \rightarrow gluconolactone + GOD(FADH_2) \qquad 3$$

Hence, upon addition of glucose to an air-saturated PBS solution, the cathodic peak current decreases because of the enzymatic reaction between the oxidized form of GOD and glucose.

Figures 4E and 4F show the typical current-time responses of different electrodes for successive addition of 0.5 mM glucose to air-saturated and stirred 0.2 M pH 7.4 PBS at -0.45 V. Each successive addition of 0.5 mM glucose results in a decrease in the steady state current, and the Ag-GOD electrode shows a sensitivity of 13.8 μA/mM and fast response time of less than 5 seconds. The calibration curve of the electrode (Fig. 4E inset) shows a linear response range from 0.014 mM to 3 mM with a correlation coefficient of 0.998. The plot of amperometric response vs. the detected glucose concentration illustrates a well-defined, typical behavior of an enzymatic kinetic reaction, while the Ag/GOD electrode has a far narrower linear range within 1 mM and higher sensitivity of 21.2 μA/mM. This may be caused by the different combination modes between silver and GOD leading to various adsorption quantity and stability on the surface of GOD. In the in situ preparation process, more GOD was covered by Ag nanoparticles effectively and greatly increased stability. In simple mixing of Ag and GOD, more GOD were absorbed on the surface of Ag particles, which let the modified electrode deliver higher sensitivity and lead to GOD gradually dissolving into PBS solution and decreased stability.

The selectivity of Ag-GOD and Ag/GOD electrodes are evaluated in the presence of common interfering species such as ascorbic acid, dopamine, and uric acid in air-saturated PBS. As shown in Fig. 4G and H, for each addition of 0.5 mM glucose the biosensors show quick response. However, no noteworthy response was observed for the addition of 0.05 mM of ascorbic acid, dopamine, and uric acid. Thus, Ag-GOD and

Ag/GOD electrodes are highly selective for glucose due to the low test potential.

The long-term stability of the two kinds of electrodes is investigated by recording the current response to 0.5 mM glucose. The current response of the Ag-GOD electrode remained about 94% of the initial response current after two weeks, while the Ag/GOD electrode only kept about 72%. The good stability of the Ag-GOD based biosensor may be attributed to the unique in situ immobilization method.

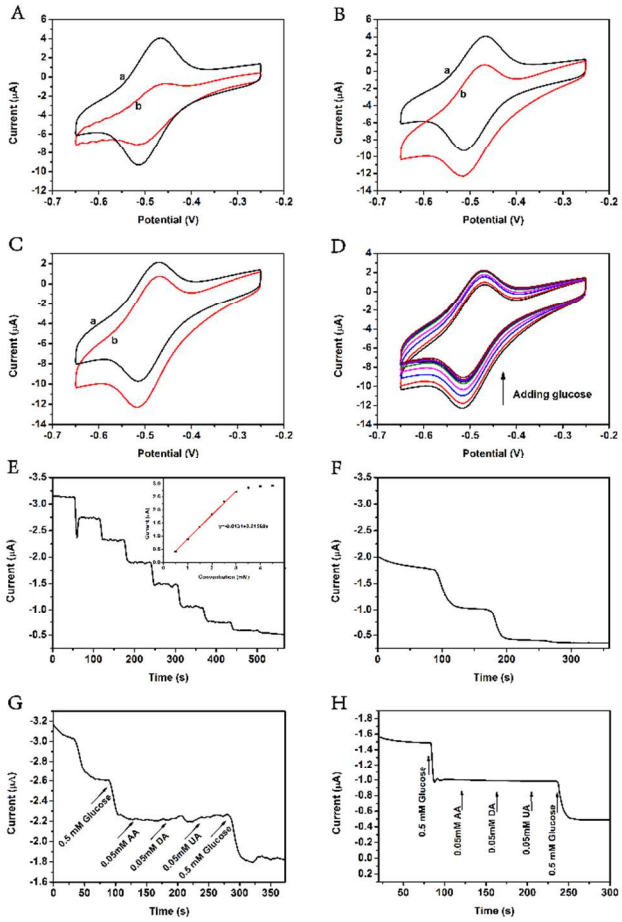

Fig. 4. (A) CV comparison of Ag-GOD/Nafion (a) and Ag/GOD/Nafion (b) electrode in N_2-saturated pH 7.4 PBS. (B) CV comparison of Ag-GOD/Nafion electrode in N_2-saturated (a) and air-saturated (b) pH 7.4 PBS. (C) The CV of Ag-GOD/Nafion electrode in air-saturated PBS without glucose (a) and with 2 mM glucose (b). (D) The CVs with an increase of 0.5 mM glucose for each time. (E) Amperometric response of Ag-GOD/Nafion electrode with successive addition 0.5 mM glucose at the potential of -0.45 V, inset is the corresponding linear relation. (F) Amperometric response of Ag/GOD/Nafion electrode with successive addition 0.5 mM glucose at the potential of -0.45 V. (G) (H) Amperometric response of Ag-GOD/Nafion and Ag/GOD/Nafion electrode with successive addition 0.5 mM glucose, 0.05 mM AA, DA and UA at the potential of -0.45 V.

The effect of silver amount in Ag-GOD composites on their electro-catalytic performance has also been observed. As shown in Fig. 5, with the increasing of Ag content in Ag-GOD composites, the intensity of redox peaks increased. But when the amount of Ag in Ag-GOD composites further increased, its redox peaks current cut down obviously, and its reversibility also become worse. These results indicated that a certain amount of silver can help GOD be used fully, and then when the amount of silver is too high, it will reduce the activity of GOD due to a high concentration of silver ions may induce a big change of the secondary structure of protein. In addition, the as-prepared glucose biosensor has been used to detect glucose in the serum samples. The glucose concentration in raw serum is 3 mM, detected by commercialized glucose meter. Adding 1 mL of serum sample into 5 mL of pH 7.4 PBS, hence the glucose concentration of the dilute serum is 0.5 mM based on glucose meter. The current response of the dilute serum was performed at -0.45 V under stirring, as shown in Fig. 5B. The concentration of glucose in the serum sample was calibrated using standard glucose solution. We can know ΔI is 0.487 mA from Fig. 5B. Based on Fig. 4E, y=-0.0131+0.9569x, the glucose concentration of the diluted serum is 0.523 mM. That means the relative standard deviations is 4.6 % compared to the standard method. So the results from the biosensor are similar to that tested by the glucose meter, indicating the as-prepared biosensor may have potential in real sample analysis.

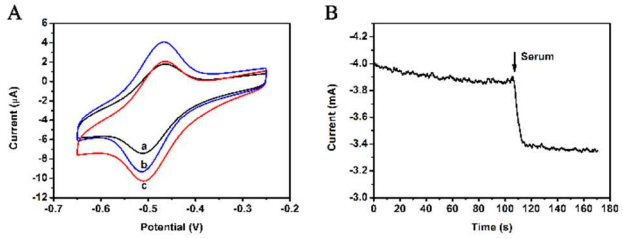

Fig. 5. (A) The cyclic voltammograms of the electrode modified with Ag-GOD composites synthesized by different amount of $AgNO_3$. (5 μL of 0.01 M $AgNO_3$ (a), 10 μL of 0.01 M $AgNO_3$ (b), 20 μL of 0.01 M $AgNO_3$ (c)). (B) Amperometric response of the diluted serum on Ag-GOD/Nafion electrode at -0.45 V under stirring.

Conclusions

A new and effective enzyme immobilization method, in situ growth metal on enzyme, is presented in our work. The experimental results indicate that Ag-GOD has good sensitivity of 13.8 μA/mM, wide detection range from 0.014 mM to 3 mM, fast response of less than 5 s, and long life. These properties demonstrate that in situ growth method is an attractive way to immobilize enzymes and exhibits facile, direct electrochemistry of GOD without any electron mediator. Moreover, this approach presents a new platform for us to develop other biosensor.

Acknowledgements

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Notes and references

Institute for Clean Energy & Advanced Materials,Faculty of Material & Energy, Southwest University, Chongqing, 400715, P. R. China
E-mail: baoshj@swu.edu.cn (S. J. Bao)

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