

A novel amperometric biosensor based on gold nanoparticles-mesoporous silica composite for biosensing glucose

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We report a novel bienzyme biosensor based on the assembly of the glucose oxidase (GOD) and horseradish peroxidase (HRP) onto the gold nanoparticles encapsulated mesoporous silica SBA-15 composite (AuNPs-SBA-15). Electrochemical behavior of the bienzyme bioconjugates biosensor is studied by cyclic voltammetry and electrochemical impedance spectroscopy. The results indicate that the presence of mesoporous AuNPs-SBA-15 greatly enhanced the protein loadings, accelerated interfacial electron transfer of HRP and the electroconducting surface, resulting in the realization of direct electrochemistry of HRP. Owing to the electrocatalytic effect of AuNPs-SBA-15 composite, the biosensor exhibits a sensitive response to H₂O₂ generated from enzymatic reactions. Thus the bienzyme biosensor could be used for the detection of glucose without the addition of any mediator. The detection limit of glucose was 0.5 μM with a linear range from 1 to 48 μM.

bienzyme electrode, mesoporous silica, glucose amperometric detection

1 Introduction

The accurate, rapid quantitative determination of glucose is of great importance in clinical chemistry, biochemistry and food analysis^[1]. In the last decades, the electrochemical enzymatic glucose sensors have attracted more attention due to their high specificity^[2]. Glucose oxidase (GOD) can catalyze the oxidation of glucose to hydrogen peroxide (H₂O₂) and gluconolactone in the presence of dissolved oxygen. However, the major challenge for the development of new biosensors for the determination of glucose is the elimination of interferences, which hindered the sensitivity of the devices. These interferences mainly come from the presence of more easily oxidizable compounds present in biological fluids than the liberated H₂O₂ produced by the enzymatic reaction. Recently, co-immobilization of horseradish peroxidase (HRP) with a H₂O₂ producing oxidase gives a biosensor with selectivity towards the

substrate of the oxidase. For example, Kulys et al.^[3] designed the first oxidase/peroxidase bienzyme electrode with a film containing HRP and GOD to determine glucose by measuring the amount of H₂O₂ produced through the peroxidase. Zambonin et al.^[4] fabricated a glucose bienzyme sensor based on HRP and GOD immobilized in an electropolymerized film. Narayanan et al.^[5] developed a bienzyme nanobiocomposite biosensor using functionalized carbon nanotubes for the detection of H₂O₂ and glucose. The coupling of several enzymes through substrate or co-substrate regeneration among these multienzymatic systems is quite promising for amplifying the electrochemical responses in biosensing

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applications.

On the other hand, SBA-15, as a type of mesoporous silica, has a large internal surface, tunable uniform pore size, chemical and thermal stability. It has attracted considerable attention in biosensor platforms for the detection of hydrogen peroxide and glucose^[6]. Recent studies have demonstrated that SBA-15 is able to improve the direct electron transfer reaction of some biomolecules, which is attributed to its mesoporous structure. Additionally, gold nanoparticles with appealing electrical properties and biocompatibility have already been used in sensor fabrication, which have been found to be able to accelerate electron exchange between electrode and protein^[7]. Therefore, immobilization of enzyme on gold nanoparticles encapsulated mesoporous SBA-15 will show certain advantages over those glucose biosensors in which enzymes are directly immobilized on bare electrode surfaces.

In this paper, the gold nanoparticles were encapsulated into the channels of mesoporous silica SBA-15 to fabricate a AuNP-SBA-15 hybrid material. This composite was further used for the immobilization of bienzyme to develop a mediator-free amperometric glucose biosensor. One of the enzymes, GOD converts glucose to gluconic acid with H_2O_2 , which is the substrate for the second enzyme, HRP. HRP is in direct electronic communication with the electrode via AuNPs-SBA-15 thus bringing about the electrocatalytic reduction of H_2O_2 , which can be further measured by amperometric method. The biosensor showed high performance characteristics with a broad detection range and a long-term stability of H_2O_2 and glucose, which implied that the proposed method provided an excellent platform for sensitive electrochemical sensing and biosensing.

2 Experimental

2.1 Chemicals

GOD (activity 250 units/mg), HRP (250 units/mg) and poly(ethylene-oxide)-poly(propylene oxide)-poly(ethyleneoxide) block copolymer $EO_{20}PO_{70}EO_{20}$ (P123) were purchased from Sigma Co. Hydrogen peroxide (30% (w/v)), $H AuCl_4 \cdot 4H_2O$ and Tetraethyl orthosilicate (TEOS) were from Shanghai Chemical Reagent Co. (Shanghai, China). (3-Aminopropyl)triethoxysilane (APTES) was obtained from Nanjing Reagent Factory (Nanjing, China). Phosphate buffer solutions (PBS, 0.1

M) with various pH values were prepared by mixing stock standard solutions of Na_2HPO_4 and NaH_2PO_4 and adjusting the pH with H_3PO_4 or NaOH. All other chemicals were of analytical grade and used without further purification.

2.2 Synthesis of SBA-15 and AuNPs-SBA-15 nanostructures

The mesoporous SBA-15 was synthesized according to the method described by Zhao et al.^[8]. The synthesis of the AuNPs-SBA-15 nanocomposite was as follows: First, the temple-free SBA-15 material was rehydrated by refluxing with water for 6 h, followed by heating at 200°C overnight. After cooling to room temperature, the sample was immersed in an ethanol solution of the APTES and refluxed at 70°C for 6 h. The functionalized SBA-15 was filtered, washed with a large amount of ethanol and dried. Subsequently, 0.4 g of functionalized SBA-15 was mixed with 6 mL of an aqueous 1 wt% $H AuCl_4$ solution at pH 2, followed by stirring for 30 min. After reaction, the mixture was filtered, washed with water, and then dried in air. The resulting yellowish solid was further reduced by the addition of 5 mL of 0.1 M KBH_4 aqueous solution and stirred for 30 min. The AuNPs-SBA-15 was collected by centrifugation and further washed with water.

2.3 Preparation of TEOS stock sol-gel solution

A homogeneous stock sol-gel solution was prepared by mixing 600 μL of ethanol, 50 μL of TEOS, 10 μL of 5 mM NaOH, and 60 μL of H_2O in a small test tube at room temperature. After being sonicated, the sol-gel was formed and stored at 4°C. The sol-gel solution was prepared freshly before each electrode modification experiment

2.4 Construction of the bienzyme bioconjugates biosensor

The bienzyme bioconjugates biosensor was fabricated as follows: First, 5 mg of AuNPs-SBA-15 composite was dispersed in 1 mL of pH 7.0 PBS containing 5 mg of HRP and 5mg of GOD, and shaken at room temperature overnight. Subsequently, 5 μL of this suspension was dropped onto the glassy carbon electrode (GCE, 3 mm in diameter) and dried in a silica gel desiccator. After 2 h, 5 μL of stock silica sol-gel solution was pipetted to cover the bioconjugates-modified GCE. Finally, the electrode was left to dry at 4°C overnight. The modified

electrode was stored under the same conditions when not used.

2.5 Apparatus and measurements

Cyclic voltammetric measurements were performed using a CHI660a workstation (Shanghai Chenhua, China). All electrochemical experiments were performed with a conventional three-electrode system, using a platinum wire as the auxiliary, a saturated calomel electrode as the reference and the modified GCE as the working electrode. Electrochemical impedance experiments were performed on a PGSTAT30/FRA2 system (Autolab, Netherlands) in 1 M KCl containing 10 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ (1:1). The impedance spectra were recorded within the frequency range of 10^{-1} – 10^5 Hz with 5 mV amplitude of the applied sine wave potential.

Fourier transform infra spectra (FT-IR) were obtained on a NEXUS 670 (Nicolet) FT-IR instrument at room temperature. Transmission electron micrographs (TEM) were recorded on a JEOLJEM 200CX transmission electron microscope, using an accelerating voltage of 200 keV. Brunauer-Emmett-teller (BET) data were collected with a Micromeritics-ASAP 2020 surface area and porosity analyzer at 77 K. The BET surface area was calculated from the linear of the BET plot. The pore-size distribution plots are obtained by using the Barrer-Jovner-Halenda (BJH) model.

3 Results and discussion

3.1 Characterization of AuNPs-SBA-15 and bienzyme bioconjugate

Mesoporous SBA-15 material has already been used as a suitable host for metal nanoparticles such as gold nanoparticles. Here, well-defined AuNPs-SBA-15 composite was synthesized by using a chemical reduction method as described in experimental section 2.2. Figure 1(a) shows the typical TEM image of the AuNPs-SBA-15 composite. It can be seen that the composite has a highly ordered hexagonal pore structure with tiny, high uniform gold nanoparticles in the channel. It is also worth noting that there is little AuNPs on the external surface. The gold particles are not all spherical, but some seem to be elongated and oriented along the channels of SBA-15. This suggests that the gold nanoparticles are in contact with the functionalized channels and the growth of the nanoparticles is restricted by the channel walls.

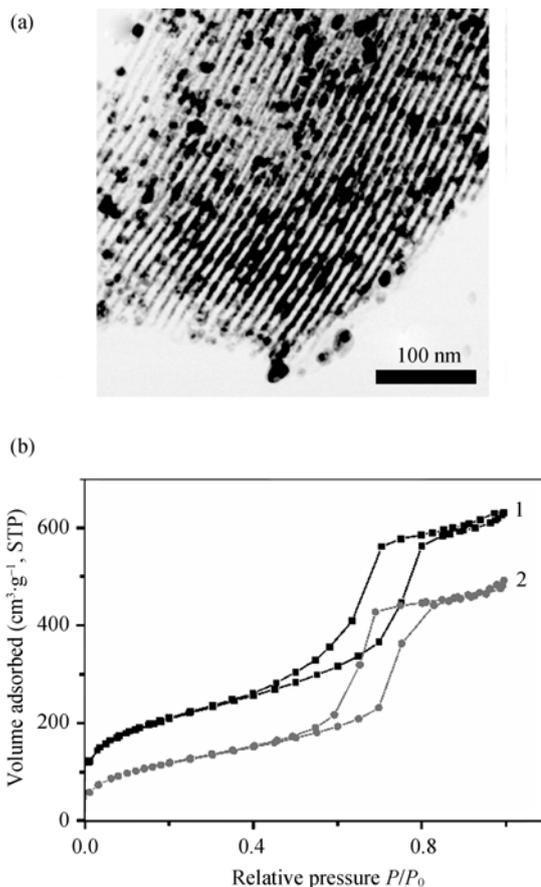


Figure 1 (a) TEM image of AuNPs-SBA-15 nanocomposite; (b) nitrogen adsorption/desorption isotherms of SBA-15 before (cure 1) and after (cure 2) encapsulation of AuNPs.

The BET method of nitrogen adsorption/desorption was further used to confirm the pore-filling effect after encapsulation of AuNPs. As shown in Figure 1(b), the isotherm exhibits a distinct hysteresis loops and sharp adsorption/desorption steps, indicating a narrow pore size distribution. Meanwhile, the shape of the N_2 adsorption/desorption isotherm of AuNP-SBA-15 is similar to that of the pure SBA-15, indicating that the mesoporous structure of SBA-15 is not destroyed during the pore-filling progress. After encapsulation of AuNPs, the BET surface area (S_{BET}) and the BJH desorption pore volume (V_{P}) decreased from 739 to 435 $\text{m}^2\cdot\text{g}^{-1}$ and from 1.20 to 0.78 $\text{cm}^3\cdot\text{g}^{-1}$, respectively, and the BJH desorption pore size (D_{P}) decreased from 6.1 to 5.7 nm mainly due to the blockage of the SBA-15 pores by AuNPs.

FT-IR spectroscopy is sensitive to the secondary structure of the protein. The profiles of the amides I and II infrared absorbance bands of enzyme can provide the detailed information on the secondary structure of

the polypeptide chain^[9]. The amide I band (1700–1600 cm^{-1}) results from the C=O stretching vibration of peptide linkages in the protein backbone. The amide II band (1620–1500 cm^{-1}) is attributed to the combination of N–H bending and C–N stretching. Figure 2(a) showed the FT-IR spectra of HRP, GOD, AuNPs-SBA-15 and GOD-HRP-AuNPs-SBA-15. It can be seen that the amide I and II bands of enzymes in the GOD-HRP-AuNPs-SBA-15 composite are located at 1655 and 1543 cm^{-1} , respectively, which are nearly the same as those obtained for native HRP (1654 and 1542 cm^{-1}) and GOD (1650 and 1542 cm^{-1}). Because previous studies showed that amides I and II would significantly diminish if enzyme was denatured, similarities of the three spectra suggest that GOD and HRP both retain the essential features of native secondary structure in GOD-HRP-AuNPs-SBA-15 composite.

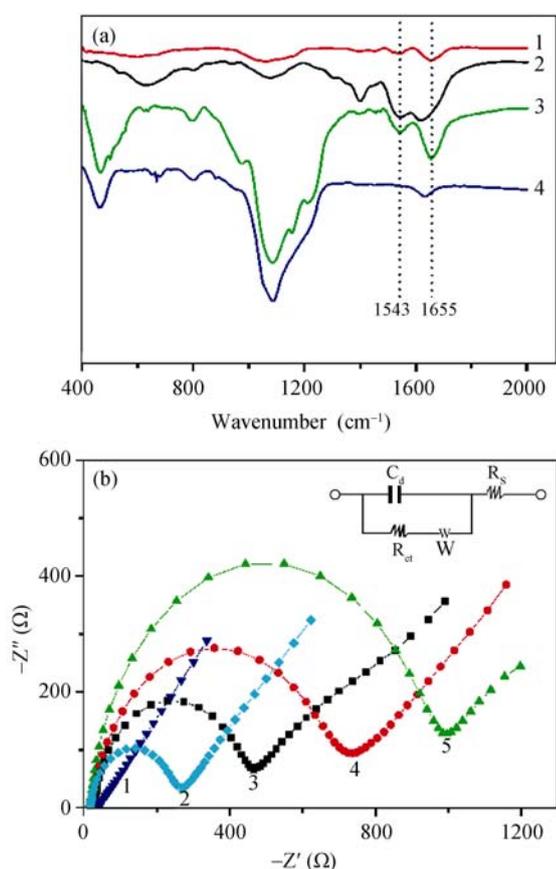


Figure 2 (a) FTIR spectra of (1) HRP, (2) GOD, (3) GOD-HRP-AuNPs-SBA-15 and (4) AuNPs-SBA-15. (b) Nyquist plots of the electrochemical impedance spectroscopy (EIS) for (1) bare GCE, (2) AuNPs-SBA-15/GCE, (3) GOD-AuNPs-SBA-15/GCE, (4) HRP-AuNPs-SBA-15/GCE and (5) GOD-HRP-AuNPs-SBA-15/GCE respectively.

Electrochemical impedance spectroscopy (EIS) was also used to monitor the fabricating process. The redox couple $\text{Fe}(\text{CN})_6^{3-/4-}$ is widely used as an electrochemical probe in the electrochemical impedance study, especially for the characterization of biomaterial-modified electrodes^[10]. Figure 2(b) showed the Nyquist plots of EIS of the modified GCEs. 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 GCE exhibits an almost straight line (curve 1) which implied the characteristic of a diffusion limiting step of the electrochemical process, while the AuNPs-SBA-15/GCE has a small increase in R_{et} (curve 2) indicates the good conductivity of the composite. When further immobilization of monoenzymes, GOD and HRP (curves 3 and 4), the values of R_{et} increased to 447 and 697 Ω , respectively, which is due to the generation of the insulating protein layer on the assembled surface. Moreover, after the immobilization of both the enzymes on the AuNPs-SBA-15 composite, the R_{et} markedly increased to about 973 Ω (curve 5) which is due to the attachment of each of the enzymes that increases the extent of insulation of the conductive support by the hydrophobic protein layers^[11].

3.2 Direct electrochemistry of GOD-HRP-AuNPs-SBA-15 modified electrode

The electrochemical behavior of the immobilized bienzyme was studied with cyclic voltammetry. No redox peak is observed for the CVs of bare GCE and AuNPs-SBA-15 modified GCE, indicating the electro-inactiveness of AuNPs-SBA-15 composite. In contrast, a pair of stable and well-defined redox peaks are observed on GOD-HRP-AuNPs-SBA-15 modified GCE with a formal potential of -0.355 V. This couple of redox peaks was attributed to the direct electrochemistry of the immobilized HRP, which is similar to the results reported^[12]. Obviously the AuNPs-SBA-15 composite provided a biocompatible microenvironment for the bienzyme to retain native structure and the AuNPs played an important role in facilitating the direct electron transfer of HRP. Figure 3 shows the cyclic voltammograms at GOD-HRP-AuNPs-SBA-15 modified GCE in N_2 -saturated pH 7.0 PBS at various scan rates. Both cathodic and anodic peak currents increased linearly with scan rates from 50 to 350 $\text{mV}\cdot\text{s}^{-1}$, which is characteristic of thin-layer electrochemistry. According to Faraday's

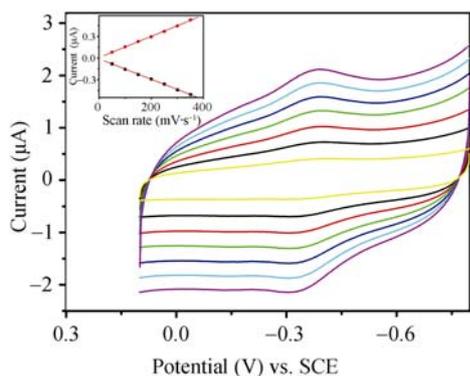


Figure 3 Cyclic voltammograms of the GOD-HRP-AuNPs-SBA-15/GCE in 0.1 M PBS (pH 7.0) with N_2 -saturated at various scan rates (from inner to outer curves: 50, 100, 150, 200, 250, 300, 350 $mV \cdot s^{-1}$). Inset is the plots of peak currents vs. scan rates.

law, an average surface coverage of enzymes was estimated to be $3.69 \times 10^{-10} \text{ mol} \cdot \text{cm}^{-2}$, which is larger than the theoretical monolayer coverage of HRP (ca. $5.0 \times 10^{-11} \text{ mol} \cdot \text{cm}^{-2}$)^[13]. The value obtained in experiments showed that a multilayer of enzyme molecules were assembled on the bienzyme bioconjugate modified electrode. Based on Laviron theory, the electron transfer rate constant (K_s) and charge transfer coefficient (α) can be made from the peak potential separation value. The calculated values for K_s and α were about 0.43 s^{-1} and 0.51, respectively.

3.3 Electrochemical oxidation of glucose at the bi-enzyme bioconjugates biosensor

The working mechanism of GOD-HRP bienzyme electrode studied in this work is depicted in Figure 4(a). In the presence of natural co-substrate O_2 , glucose oxidase catalyses the oxidation of glucose to gluconic acid, with production of H_2O_2 (eqs. (1) and (2)). The H_2O_2 then serves as substrate for HRP, producing an unstable intermediate HRP(ox), which subsequently has a direct electronic transfer with the electrode through AuNPs-SBA-15 composite (eqs. (3) and (4)).



In total:

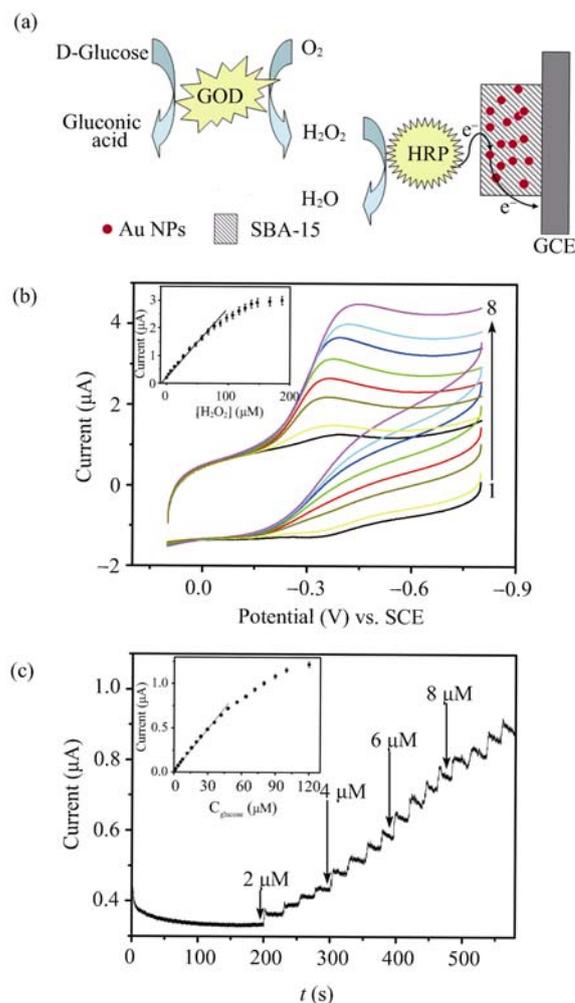
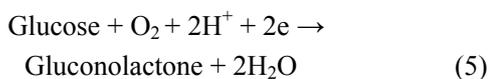


Figure 4 (a) The reaction scheme of the GOD-HRP-AuNPs-SBA-15/GCE for detection of glucose in solution. (b) Cyclic voltammograms of the GOD-HRP-AuNPs-SBA-15/GCE at scan rate of $200 \text{ mV} \cdot \text{s}^{-1}$ in 0.1 M pH 7.0 PBS solution with (1) 0, (2) 1.3, (3) 27.3, (4) 47.3, (5) 67.3, (6) 97.3, (7) 127.3 and (8) 147.3 μM H_2O_2 . Inset: The calibration curve of the electrocatalytic current on the concentration of H_2O_2 . (c) Amperometric response of GOD-HRP-AuNPs-SBA-15/GCE upon successive additions of glucose to 0.1 M pH 7.0 PBS at -0.45 V . Inset: The calibration curve of the electrocatalytic current on the concentration of glucose.

Prior to the study of the performance of the bienzyme electrode for glucose detection, we first characterized the electrocatalytic reactivity of GOD-HRP-AuNPs-SBA-15 modified electrode toward H_2O_2 . As shown in Figure 4(b), in the absence of H_2O_2 , two typical reversible redox waves were observed (curve 1). Nevertheless, upon addition of H_2O_2 , the cathodic peak was greatly enhanced, while the corresponding anodic peak decreased, suggesting that an electrocatalytic reduction of H_2O_2 occurred. Furthermore, the reduction peak current increased with the increase of H_2O_2 concentration, which indicated that the HRP could retain its good bio-

activity on the bienzyme electrode. The electrocatalytic curve of the bienzyme electrode to H_2O_2 concentration obtained with cyclic voltammetry was shown in the inset of Figure 4(b). The biosensor showed a linear relationship for H_2O_2 from 0.3 to 67.3 μM . The linear regression equation was $y = 0.027x + 0.20 \mu\text{A}$, with a correlation coefficient of 0.996. From the slope of $0.027 \mu\text{A}\cdot\mu\text{M}^{-1}$, the detection limit was estimated to be 0.1 μM at 3σ . The apparent Michaelis-Menten constant (K_{Mapp}) was 0.12 mM, which could be calculated from the electrochemical version of the Lineweaver-Burk equation. The K_{M} value for this bienzyme biosensor was smaller than that of 0.16 mM for HRP-Nafion-cysteine modified gold electrode^[14] and 0.93 mM for a HRP entrapped chitosan/silica sol-gel hybrid membranes^[15]. Therefore, the present study clearly shows that the biosensor exhibits a high catalytic activity to H_2O_2 .

The glucose biosensors are generally based on the detection of the oxidation signal of hydrogen peroxide or the reduction signal of dissolved oxygen. The excellent catalytic activity to H_2O_2 at the bienzyme electrode makes the bienzyme electrode extremely attractive for amperometric detection of glucose. Figure 4(c) illustrates a steady-state response of the biosensor to additions of glucose to a stirred PBS at an applied potential of -0.45 V . Upon addition of glucose, the current increased steeply to 95% of steady-state current in less than 10 s, indicating the AuNPs-SBA-15 composite created a fast diffusion of substrate from the bulk solution

to the bienzyme. The calibration curve in the inset of Figure 4(c) showed a good linear relationship with the concentration of glucose from 1 to 48 μM and the detection limit was estimated to be 0.5 μM at 3σ . These results indicated that the AuNPs-SBA-15 composite greatly increased the immobilization of bienzyme and provided a favorable microenvironment for bienzyme to retain bioactivity.

The bienzyme biosensor showed a good long-term stability and retained 91% of its response to glucose after two weeks storage. Reproducibility of the biosensor system was evaluated from the response for 0.1 mM glucose at six different biosensors and an acceptable RSD. value of 6.0 % was observed. The good long-term stability can be attributed to the efficient biocompatibility and stability of the hybrid material.

4 Conclusions

In this paper, we present the development of a bienzyme biosensor based on the immobilization of bi-enzymes GOD and HRP on AuNPs-SBA-15 composite. The bienzyme biosensor showed a fast direct electron transfer of HRP and excellent catalytic activity to hydrogen peroxide. Additionally, the biosensor also displayed high performance characteristics with a broad detection range and a good long-term stability. Thus, this mesoporous hybrid material provides a novel and promising platform for the study of electron transfer of proteins and the development of biosensors.

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