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Direct electrochemistry of glucose oxidase immobilized on a hexagonal mesoporous silica-MCM-41 matrix

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Abstract

The direct electrochemistry of glucose oxidase (GOD) immobilized on a hexagonal mesoporous silica modified glassy carbon electrode was investigated. The adsorbed GOD displayed a pair of redox peaks with a formal potential of −417 mV in 0.1 M pH 6.1 phosphate buffer solution (PBS). The response showed a diffusion-controlled electrode process with a two-electron transfer coupled with a two-proton transfer reaction process. GOD immobilized on a hexagonal mesoporous silica retained its bioactivity and stability. In addition, the immobilized GOD could electrocatalyze the oxidation of glucose to gluconlactone by taking ferrocene monocarboxylic acid (FMCA) as a mediator in N_2 saturated solutions, indicating that the electrode may have the potential application in biosensors to analyze glucose. The sensor could exclude the interference of commonly coexisted uric acid, *p*-acetaminophenol and ascorbic acid and diagnose diabetes very fast and sensitively. This work demonstrated that the mesoporous silica provided a novel matrix for protein immobilization and the construction of biosensors.

Keywords: Biosensors; Glucose oxidase; Hexagonal mesoporous silica; Chemically modified electrode; Direct electrochemistry; Glucose

1. Introduction

GOD has been extensively used to monitor the blood glucose levels in diabetics for its catalytic ability to glucose. It is a structurally rigid glycoprotein with a molecular weight of 152,000–186,000 Da, and consists of two identical polypeptide chains, each containing a flavin adenine dinucleotide (FAD) redox center, which are deeply embedded in the apoenzyme. It catalyzes the electron transfer from glucose to gluconlactone. The distance between its two FAD/$FADH_2$ centers and the electrode surface is so long that direct electron transfer from the enzyme to the electrode is difficult to be realized. Many attempts have been done in order to improve the communication between active site of enzyme and electrode. The direct electron transfer of immobilized GOD has been achieved on platinum or sputtered platinum [1,2], gold [3], glassy carbon [4], carbon paste [5,6], graphite surface [7], carbon nanotubes [8], carbon nanotubes/chisol [9] and CdS nanoparticles [10] by amperometric and cyclic voltammetric techniques. Since GOD activity is quite sensitive to the environment and easy to destroy following the electron transfer, although a variety of materials can promote the electron transfer between GOD and electrode and can be used as the matrixes to immobilize GOD, a kind of biological compatibility material needs to be provided.

Recently, a series of inorganic porous materials such as clay [11], montmorillonite [12–14], porous alumina [15] and sol–gel matrix [16] have been proven to be promising as the immobilization matrices because of their high mechanical, thermal, and chemical stability as well as good adsorption and penetrability. The unique structural and catalytic properties of mesoporous molecular sieves for structuring an electrochemical/electron transfer environment and resistance to biodegradation have also attracted considerable attention [17]. As a protein immobilizing matrix, mesoporous molecular sieves can incorporate proteins through physical or chemical action and it has a good adsorption due to its large specific surface area. A mesoporous molecular sieves with the appropriate dimensions and functionalization adjacent to the enzyme redox center could

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act as a current nanocollector and as an electron relay to a macroelectrode. The incorporation of mesoporous molecular sieves into redox enzymes could provide an active biomaterial [18]. Herein, we choose a hexagonal mesoporous silica (MCM-41), which processes a porous size of nanoscale dimension to make it more suitable for enzyme immobilizing. This work demonstrates the application of MCM-41 to GOD immobilization, direct electron transfer and biosensing. The immobilized GOD shows a direct electrochemistry and exhibits a good electrocatalytic behavior to glucose. The prepared sensors can be used for the determinations of glucose.

2. Experimental

2.1. Reagents

Glucose oxidase (GOD, EC 1.1.3.4, 35.3 units/mg. Type II from *Aspergillus niger*) and β-D-(+)-glucose were purchased from Sigma and used as received. Nafion (10% in methanol with equivalent weight of about 1100) was obtained from Aldrich and was diluted to 5% with H_2O before use. Ferrocene monocarboxylic acid (FMCA, 97%, Aldrich) was used as received. All other chemicals were of analytical grade and were used without further purification. All solutions were made up with twice-distilled water.

2.2. Immobilization of GOD

MCM-41 was prepared following a recipe similar to that reported by Beck [19]. The specific surface area and pore volume obtained by the N_2 adsorption data and calculated by the BET method of Barrett et al. [20] and the diameter of the MCM-41 derived from the adsorption branch using the BJH method, were listed in Table 1.

30 mg of MCM-41 was dispersed into 10 mL 0.1 mM GOD (in pH 6.1 PBS) solution. The mixture was stirred for 3 h to obtain a suspension. 100 μL of the obtained suspension was then mixed with 5 μL of 10% Nafion solution to produce GOD/MCM-41/Nafion colloid that was used for the following work.

2.3. Electrode modification

The glassy carbon electrodes (GCE, 3 mm in diameter) were polished to a mirror-like finish with 1.0, 0.3 and 0.05 μm alumina slurry (Beuhler) followed by rinsing thoroughly with doubly distilled water. The electrodes were successively sonicated in 1:1 nitric acid, acetone and doubly distilled water, and then allowed to dry at room temperature. The real area of the pretreated GCE was 0.092 cm^2 determined by the slope of the plot of the anodic peak current of 1.0 mM $K_3[Fe(CN)_6]$ in 0.1 M KCl *vs*. the square root of scan rate. 3 μL GOD/MCM-41/Nafion colloidal solution was dropped on the pretreated GCE surface and allowed to dry under ambient condition for 3 h. After the modified electrode was rinsed with doubly distilled water twice or thrice, GOD/MCM-41/Nafion/GCE was obtained. When not in use the electrode was stored in 0.1 M pH 6.1 PBS at 4 °C.

Table 1
Pore Characterization of MCM-41

A_{BET} (m^2/g)	V_{total} (cm^3/g)	a_0 (nm)	D(nm)	L (nm)
622	0.78	5.48	3.36	2.12

$A_{BET,}$ total specific surface area; V_{total}, total mesopore volume; a_0, lattice parameter; D, mesopore diameter; L, wall thickness.

2.4. Apparatus and measurements

The scanning electron microscopic (SEM) images of MCM-41 and GOD/MCM-41 were obtained with a JEOL JSM-5610LV scanning electron microscope (Japan). Cyclic voltammetric and amperometric measurements were performed on CHI660 electrochemical workstation (CH Instruments, USA). All electrochemical experiments were carried out in a cell containing 5.0 mL 0.1 M PBS at room temperature (25±2 °C) and using a platinum wire as auxiliary, a saturated calomel electrode as reference and the GOD/MCM-41/Nafion/GCE as working electrodes. All solutions were deoxygenated by bubbling highly pure nitrogen for at least 20 min and maintained under nitrogen atmosphere during measurements. The amperometric experiments were carried out by applying potential of 350 mV for β-D-(+)-glucose on a stirred cell at 25± 2 °C. The sensor responses were measured as the difference between total and residual currents.

3. Results and discussion

3.1. Morphologies of MCM-41 and GOD/MCM-41 films

The morphologies of GOD, MCM-41 and GOD/MCM-41 films were characterized by scanning electron microscopy (SEM). From the SEM of GOD, we can see GOD is easy to aggregate and after drying, the membrane is easy to rupture. The SEM of MCM-41 film displays a chemically clean uniform porous structure. The aggregates of the MCM-41 matrix on electrode surface are well distributed (Fig. 1b). This uniform porous structure results in a high enzyme loading. When GOD is immobilized in the MCM-41 matrix, the SEM displays a uniform and regular dense distribution for the GOD domains with a good reproducibility (Fig. 1c). Energy dispersive spectroscopy (EDS) shows that there is no N element in the MCM-41 film while N element appears on GOD/MCM-41 film which indicates GOD is indeed immobilized in MCM-41. The porous structure of the MCM-41 film makes the immobilized enzyme easy to be accessed by its substrate and brings out a good performance of the modified electrode.

3.2. Direct electron transfer of GOD/MCM-41/Nafion/GCE

FAD, a part of the GOD molecule, is known to undergo redox reaction where two protons and two electrons are released or taken up [5,7]. According to the conclusion of Ianniello et al. [7], the electrochemistry response of GOD immobilized on the

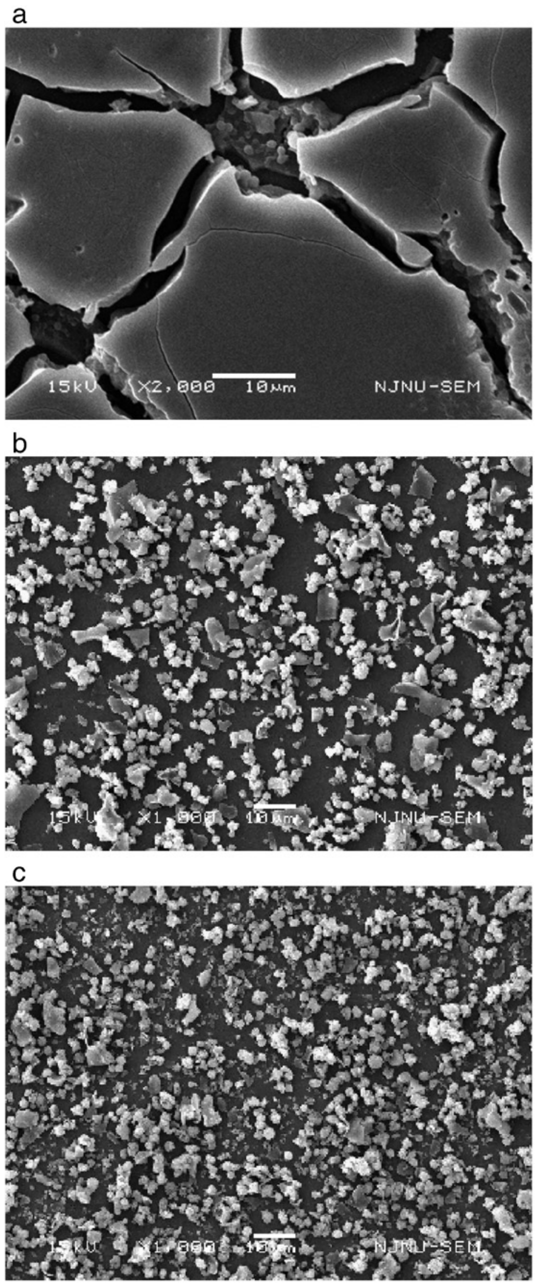

Fig. 1. Scanning electron micrographs of glassy carbon electrodes coated with GOD (a), MCM-41 film (b) and GOD/MCM-41 film (c).

heterogeneous surface is due to the redox of FAD. Fig. 2 shows the cyclic voltammograms of different electrodes in 0.1 M pH 6.1 PBS at 100 mV/s. No redox peak is observed at MCM-41/Nafion/GCE, which indicates MCM-41 is electroinactive in the potential window. GOD/Nafion/GCE also shows no peaks because in GOD, FAD is deeply seated in a cavity and therefore it is not easily accessible for conduction of electrons to the electrode surface. The GOD/MCM-41/Nafion/GCE exhibits a couple of stable redox peaks that are attributed to the redox of immobilized GOD which indicates MCM-41 plays an important role in facilitating the electron exchange between the electroactive center of GOD and GCE.

Our previous reports [21–23] used HMS, another kind of mesoporous molecular sieves to undergo the direct electron transfer of horseradish peroxidase (HRP), hemoglobin (Hb) and myoglobin (Mb) and demonstrated that the presence of HMS improved the direct electron transfer greatly between the electrode and the immobilized enzyme which indicated mesoporous molecular sieves could promote the electron transfer rate.

The mechanism of how the silica facilitated the electron transfer between the enzyme and the electrode, to our best knowledge, up to date fewer reports have discussed. The main reason might be the electrostatic interaction such as hydrogen bonding and hydrophilic attraction between GOD and MCM-41 [24,25]. The interaction between GOD and MCM-41 is much stronger than that between GOD and GCE due to the presence of SiOH groups on the external surface of MCM-41 [26]. The exact mechanism deserves further study.

The anodic and cathodic peak potentials of the immobilized GOD were at −397 and −436 mV, respectively. The formal potential was −417 mV near the standard electrode potential of −460 mV (*vs.* SCE) for FAD/FADH$_2$ at pH 7.0 (25.8 °C) [27], suggesting that most GOD molecules preserved their native structure after the adsorption process [28]. It is known that enzyme can be fixed in the pores of mesoporous materials by simply immersing the mesoporous materials in the enzyme solution [29,30]. However, the pore diameter of MCM-41 is 3.36 nm is smaller than the size of GOD (about 4–6 nm). Thus GOD can not intercalate into the mesopores of MCM-41. Therefore, the GOD should be immobilized on the external surface of MCM-41 by physical adsorption. Another evidence came from the formal potential. The formal potential of the adsorbed GOD (−417 mV) was more positive than −460 mV of native GOD in solution [27]. This was due to the presence of many acidic SiOH groups on the external surface of MCM-41 [26], which were positive-charged and made the oxidation of GOD more difficult thermodynamically.

The effect of scan rate on electrochemistry of the immobilized GOD was shown in Fig. 3. With an increasing scan rate, the anodic

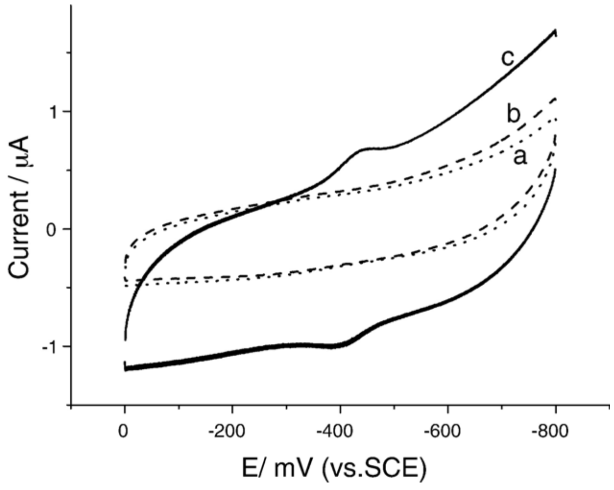

Fig. 2. Cyclic voltammograms of MCM-41/Nafion/GCE (a), GOD/Nafion/GCE (b), and GOD/MCM-41/Nafion/GCE (c) in 0.1 M pH 6.1 PBS at 100 mV/s.

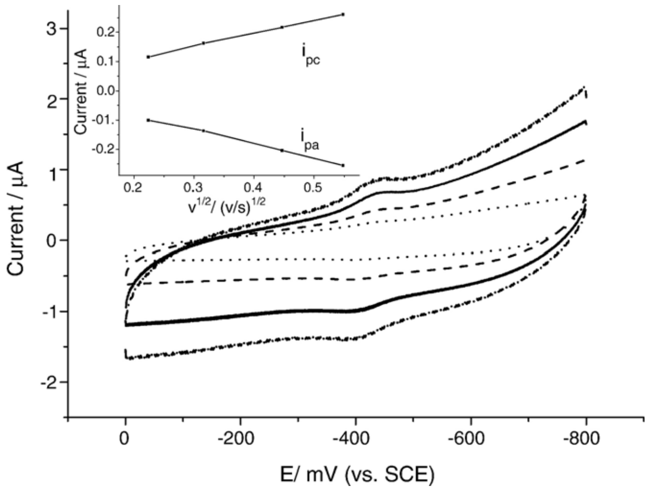

Fig. 3. Cyclic voltammograms of GOD/ MCM-41/Nafion/GCE in pH 6.1 PBS at 50, 100, 200 and 300 mV/s (from inner to outer). Inset: plots of peak currents *vs.* $v^{1/2}$.

peak potential of GOD shifted to a more positive value and the cathodic peak potential shifted in a negative direction. The redox peak currents were proportional to the square root of scan rate, $v^{1/2}$ (inset in Fig. 3), indicating a diffusion-controlled behavior [31]. The heterogeneous electron transfer rate constant was obtained from the Nicholson's equation [32], the peak-to-peak separation and the diffusion coefficient of 4.94×10^{-7} cm^2 s^{-1} [33]. The average heterogeneous electron transfer rate constant was calculated to be $(4.75\pm0.10)\times10^{-3}$ cm s^{-1}. The small peak-to-peak separation of 39 mV was observed at 100 mV/s, indicating a fast electron transfer rate which resulted from the strong interaction between GOD molecules and MCM-41. Thus, MCM-41 could provide a microenvironment for GOD to undergo facile electron transfer reactions.

Cyclic voltammogram of GOD/MCM-41/Nafion/GCE shows a strong dependence on solution pH (Fig. 4). Obviously, the maximum current response occurs at pH 6.1. An increase in solution pH causes a negative shift in both cathodic and anodic peak potentials. Plot of the formal potential *vs.* pH (from 5 to 8.0) produces a line with the slope of −56.75 mV/pH (inset in Fig. 4), which is close to the expected value of −58.0 mV/pH, indicating two protons and two electrons are involved in the electron transfer process.

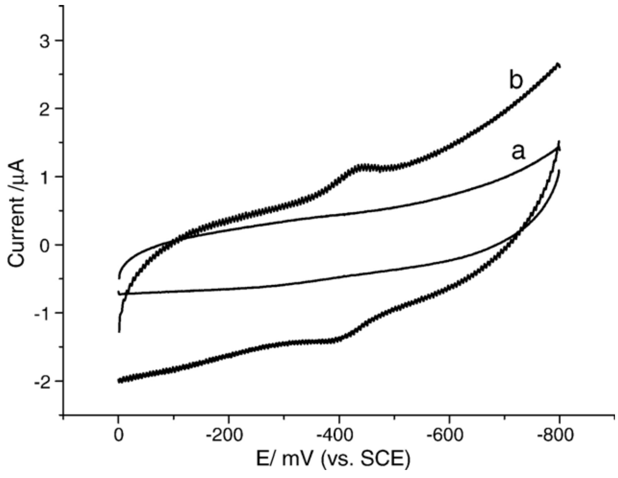

Fig. 5. Cyclic voltammograms of the GOD/MCM-41/Nafion/GCE in 0.1 M PBS (pH 6.1) with (a) and without (b) incubation in 3 M guanidine hydrochloride overnight. The scan rate is 100 mV/s.

Treatment of the GOD with guanidine hydrochloride solution can easily strip the FAD active center from the GOD molecule and remove the adsorbed GOD from the electrode surface [34]; it is relatively ineffective in removing adsorbed free FAD from the electrode surface [34]. After incubation of GOD/MCM-41/Nafion/GCE in 3 M guanidine hydrochloride solution overnight, the redox peak disappears (Fig. 5) suggesting that the redox peaks in curve b of Fig. 5 should be ascribed to the redox reaction of the FAD bound to the GOD [34] and not to free FAD.

3.3. Bioelectrocatalytic oxidation of glucose

Although the direct electron transfer of GOD has been achieved, some mediators have still been used in order to

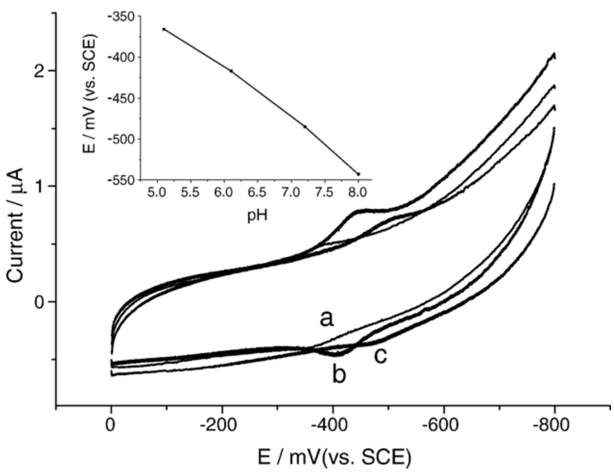

Fig. 4. Cyclic voltammograms of GOD/MCM-41/Nafion/GCE in 0.1 M pH 5.1 (a), 6.1 (b) and 7.2 (c) PBSs at 100 mV/s. Inset A: plot of peak potential *vs.* pH.

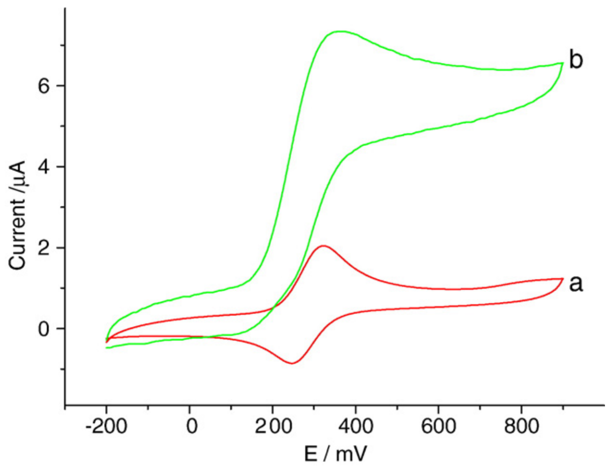

Fig. 6. Cyclic voltammograms of GOD/MCM-41/Nafion/GCE in 0.1 M PBS (pH 6.1) containing 0.2 mM FMCA in the absence (a) and presence (b) of 3.0 mM glucose.

enhance the electron transfer rate between GOD and electrode [8,9]. Fig. 6 shows cyclic voltammograms of the GOD/MCM-41/Nafion/GCE in N_2-saturated, pH 6.1 PBS containing 0.2 mM FMCA as the mediator. The pair of peaks ascribe the redox behaviors of FMCA. When 3.0 mM glucose is added into the solution, the anodic peak current increases significantly which demonstrates that GOD/MCM-41/Nafion/GCE can electrocatalyze the oxidation of glucose using FMCA as mediator in N_2 saturated solutions. These results can be explained from the following equations:

$$\text{glucose} + \text{GOD(ox)} \rightarrow \text{gluconolactone} + \text{GOD(red)} \qquad (1)$$

$$\text{GOD(red)} + 2\text{FMCA}^{+} \rightarrow \text{GOD(ox)} + 2\text{FMCA} + 2\text{H}^{+} \qquad (2)$$

$$2\text{FMCA} \rightarrow 2\text{FMCA}^{+} + 2\text{e}^{-} \qquad (3)$$

The FMCA mediated biosensor is sensitive to the presence of dissolved oxygen. So, N_2 saturated PBS is used as the electrolyte of the biosensor.

Fig. 7 shows a typical current–time plot for the sensor at 350 mV on successive addition of 5 μL 0.32 M glucose in 5.0 mL pH 6.1 PBS. When an aliquot of glucose is added into the buffer solution, the oxidation current rises steeply to reach a stable value. The sensor achieves 90% of steady-state-current in less than 10 s. Such a short response time indicates a fast electron exchange between GOD and its substrate proving further that the MCM-41 matrix is promising for the construction of biosensors due to its porous structure.

The calibration curve of the sensor shows a linear range from 0.32 to 15.12 mM for glucose response with a correlation coefficient of 0.9995. It is well known that the diabetic glucose concentration is above 7.0 mM, which indicates that this sensor can diagnose diabetes very fast and sensitively. This linear range is much wider than the range of 0.04 to 0.28 mM for GOD immobilized on colloidal gold modified carbon paste electrode [6], 0.5 to 11.1 mM for GOD at a CdS nanoparticles modified electrode [35] and 0 to 7.8 mM for GOD at a carbon nanotubes/chitosan matrix [36]. The detection limit is 0.18 mM at 3σ. Although it is higher than those for many other biosensors [36,37], it does not affect the diagnoses of diabetes.

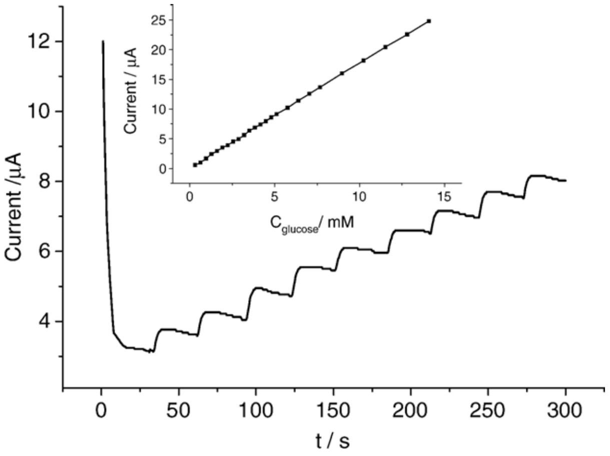

Fig. 7. Amperometric response of GOD/MCM-41/Nafion/GCE at 350 mV upon successive additions of 5 μL 0.32 M glucose to 5.0 mL 0.1 M pH 6.1 PBS containing 0.2 mM FMCA. Inset: calibration curve of glucose sensor.

At high glucose concentrations a platform response is observed, showing a characteristic pattern of the Michaelis–Menten kinetic mechanism. The apparent Michaelis–Menten constant (K_M^{app}), a reflection of the enzymatic affinity, is calculated to be 5.2 ± 0.4 mM according to the Lineweaver–Burk equation [38]. This value is much lower than 6.34 mM for GOD entrapped in titania sol–gel [39], 22 mM for GOD entrapped in copolymer modified silica sol–gel [40], 10.3 mM at the Pt/PB/GOD-Pan [41] and 25.3 mM at the GOD-polypyrrole [42] indicative of the higher affinity of GOD immobilized on MCM-41 to glucose.

The influences of foreign species were investigated by analyzing a standard solution of 1 mM glucose, to which interfering species were added. 0.3 mM uric acid, 0.1 mM *p*-acetaminophenol did not cause any observable interference to the sensor response to glucose, and only ascorbic acid at the concentration of 0.1 mM produced the relative response of about 6.0%, indicating these species coexisting in the sample matrix did not affect the determination of glucose. The mesoporous molecular sieves film was of a porous structure. Ascorbic acid could diffuse through the film to the electrode surface and be oxidized there.

3.4. Stability and reproducibility of the glucose sensor

The direct electrochemistry of the GOD/MCM-41/Nafion/GCE could retain the constant current values upon the continuous cyclic sweep over the potential range from −800 mV to 0 mV at 100 mV/s. After more than 300 successive measurements the immobilized GOD/MCM-41/Nafion/GCE only lost 9.7% of its initial activity. Thus, MCM-41 particles were very efficient for retaining the electrocatalytic activity of GOD and preventing it from leaking out of the sensor.

The fabrication reproducibility of ten electrodes, made independently, showed an acceptable reproducibility with a relative standard deviation of 4.3% for the current determined at a glucose concentration of 1.0 mM. With one sensor, the mean steady-state current was 1.7 μA with a relative standard deviation of 3.2% for six determinations at a glucose concentration of 1.0 mM.

In addition to good reproducibility, MCM-41 membrane imparted a good long-term stability to the glucose biosensor. The storage stability of glucose biosensor stored in 0.1 M pH 6.1 PBS was examined by checking periodically its relative response currents in PBS containing 1.0 mM glucose. After a storage period of 3 months in 0.1 M pH 6.1 PBS the biosensor showed an 11% loss of activity.

The recoveries for the assay of 1.0–3.0 mM glucose were between 95.3 and 104.2% for eight detections.

4. Conclusions

GOD can be effectively immobilized on MCM-41 mesoporous molecular sieve particles to produce a fast direct electron transfer. At GOD/MCM-41/Nafion/GCE the cyclic voltammogram exhibits a pair of redox peaks corresponding to a diffusion-controlled electrode process with two-proton, two-electron transfer. MCM-41 mesoporous molecular sieves retain the activity of the immobilized GOD and facilitates the electron exchange between GOD and electrode. The immobilized GOD displays a high affinity and sensitivity to glucose. The sensor shows a good reproducibility and stability. MCM-41 mesoporous molecular sieves provides an efficient matrix for developing biosensors and investigating direct electron transfer of proteins.

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