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Development of amperometric glucose biosensor through immobilizing enzyme in a Pt nanoparticles/mesoporous carbon matrix

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Abstract

Pt nanoparticles were deposited on mesoporous carbon material CMK-3. Glucose oxidase (GOx) was immobilized in the resulting Pt nanoparticles/mesoporous carbon (Pt/CMK-3) matrix, and then the mixture was cast on a glassy carbon electrode (GCE) using gelatin as a binder. The glucose biosensor exhibited excellent current response to glucose after cross-linking with glutaraldehyde. At 0.6 V (*vs.* SCE) the response current was linear to glucose concentration in the range of 0.04–12.2 mM. The response time (time for achieving 95% of the maximum current) was 15 s and the detection limit (S/N = 3) was 1 μM. The Michaelis–Menten constant (K_m^{app}) and the maximum current density (i_{max}) were 10.8 mM and 908 μA cm^{-2}, respectively. The activation energy of the enzymatic reaction was estimated to be 22.54 kJ mol^{-1}. The biosensor showed good stability. It achieved the maximum response current at about 52 °C and retained 95.1% of its initial response current after being stored for 30 days. In addition, some fabrication and operation parameters for the biosensor were optimized in this work. The biosensor was used to monitor the glucose levels of serum samples after being covered with an extra Nafion film to improve its anti-interferent ability and satisfied results were obtained.
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Keywords: Mesoporous carbon; Pt nanoparticles; Glucose oxidase; Glucose; Biosensor; Amperometry

1. Introduction

Nano-materials have been used widely in many fields in the past years [1–3]. One of their important functions is catalysis; especially noble metal nanoparticles present high catalytic activity for many chemical reactions. Because nano-materials also have good biocompatibility, they are used to immobilize biomolecules for the fabrication of biosensor, etc. Pt nanoparticles have been widely adopted to prepare biosensors due to their excellent catalytic action to the oxidation of H_2O_2 [4–6]. To construct a Pt nanoparticle-based biosensor, both Pt nanoparticles and enzyme should be loaded in an appropriate matrix. The matrix should be able to load many Pt nanoparticles and make them highly dispersed. Meantime, it can immobilize enzyme molecules effectively. Recently, various materials were tested for Pt nanoparticles loading and then were used for the fabrication of biosensors, such as carbon nanotubes [4–10], polyaniline [11], silicates [10,12] and so on. Among them carbon nanotubes attracted much attention. For example, Tang et al. developed an amperometric glucose biosensor based on the adsorption of glucose oxidase (GOx) at a platinum nanoparticles–carbon nanotubes modified glassy carbon electrode (GCE) [6]. The biosensor showed a large linear response range (0.1–13.5 mM glucose), short response time (within 5 s) and high stability. Besides, Zhou et al. reported a glucose biosensor, which was based on the immobilization of GOx on Pt nanoparticles dispersed in nano-fibrous polyaniline [11]. The biosensor exhibited linear response to glucose in the range from 2×10^{-3} to 12 mM and the response time was 7 s.

Mesoporous materials have well-ordered pore structure, high specific surface area and narrow pore size distribution [13,14]. Therefore, they are promising materials for the loading of nanoparticles and biomolecules. When nanoparticles are loaded on mesoporous materials, they are likely to be dispersed well and exhibit controlled size as they can be confined in the pores to some extent [15]. It was reported that Pt nanoparticles deposited on mesoporous carbon were highly dispersed and presented high

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catalytic activity [16,17]. In addition, in the pores of mesoporous materials biomolecules generally display good stability due to some folding forces of pores [18,19]. For example, lysozyme [20], cytochrome *c* [21], and glucose oxidase [22] were immobilized on mesoporous carbon materials with large loading amounts and high stability.

Mesoporous carbon materials possess better electron conductivity and higher thermal, mechanical and water stability than other mesoporous materials [23–25]. Hence, mesoporous carbon materials have more potential in the construction of electrochemical biosensors. In this work, a glucose biosensor based on glucose oxidase immobilized on a Pt nanoparticles/mesoporous carbon CMK-3 (Pt/CMK-3) matrix has been fabricated. The biosensor displays good catalytic activity and rapid response to glucose, and it also shows high stability. When a Nafion film is covered on it, the resulting enzyme electrode can be used to monitor glucose level of human blood.

2. Experimental

2.1. Reagents

Glucose oxidase (GOx, 146,000 U g^{-1}) was purchased from Sigma. Gelatin and polyvinyl alcohol (PVA, average degree of polymerization, 1750 ± 50) came from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Glutaraldehyde (25%) was purchased from Jingyu Fine Chemicals Corporation (Tianjin, China). Other chemicals used were of analytical grade and the solutions were prepared with double-distilled water.

2.2. Synthesis of Pt/CMK-3

Mesoporous carbon material CMK-3 (Carbon Molecular Sieve Korean Advanced Institute of Science and Technology No. 3) was prepared according to literature [26]. Mesoporous silica material SBA-15 (Santa Barbara Material No. 15) was used as template and sucrose as carbon source. The mesoporous carbon material with high loading of Pt nanoparticles was prepared following literature [27]. In brief, H_2PtCl_6 was dissolved in water. CMK-3 was preheated at 110 °C in air and then poured into the H_2PtCl_6 solution. The weight ratio of Pt to C was controlled according to the target metal loaded. After ultrasonic agitating for 30 min, the suspension was heated until it became slurry. The slurry was left overnight, and then further dried at 110 °C in an oven. The resulting agglomerate was ground in an agate mortar, placed in a glazed ceramic boat and then heated at 120 °C for 2 h in a tube furnace with H_2 atmosphere. Finally, the powder was cooled to room temperature in argon atmosphere. In this work, a Pt/CMK-3 composite material with 20 wt% Pt nanoparticles was synthesized. The morphology of Pt/CMK-3 was characterized with transmission electron microscopy (TEM, JEM-2010FEF, Japan) using an accelerating voltage of 200 kV.

2.3. Preparation of modified electrodes

Before modification, the bare glassy carbon electrode was polished to mirror smooth with 0.05 μm Al_2O_3 slurry, rinsed with water, and then ultrasonicated in water bath. Pt/CMK-3 and GOx were added into 0.6% gelatin aqueous solution. Their final concentrations were 5 and 8 mg ml^{-1}, respectively. The mixture was stirred for 10 min to obtain homogenous suspension. Three microliters of the resulting suspension was transferred onto the GCE surface, and let it dry in air. Then the electrode was immersed in 2.5% glutaraldehyde solution for 10 min in order to induce chemical cross-linking. Before use, the enzyme electrode was rinsed with buffer solution for 10 min to remove the enzyme not firmly immobilized. The obtained enzyme electrode was noted as Pt/CMK-3-GOx-gelatin/GCE. For comparison, a CMK-3-GOx-gelatin/GCE was prepared through similar way. The enzyme electrodes were stored in pH 7.0 phosphate buffer solution (PBS) at 4 °C when they were not used.

In addition, to study the electrocatalytic behavior of CMK-3 and Pt/CMK-3 to H_2O_2, CMK-3 and Pt/CMK-3 films modified electrodes were prepared. In brief, CMK-3 or Pt/CMK-3 was dispersed in *N*,*N*-dimethylformamide to prepare 3 mg ml^{-1} suspension. Then, 20 μl suspension was mixed with 2 μl 3% PVA aqueous solution. Three microliters of the resulting colloid solution was transferred onto the cleaned GCE surface, and the solvent was evaporated in air. Then CMK-3-PVA/GCE and Pt/CMK-3-PVA/GCE were obtained. Here, PVA was chosen as binder, because the modified electrodes were unstable when gelatin was used.

2.4. Methods

All electrochemical experiments were performed on a CHI 660A electrochemical workstation (CH Instru. Co., Shanghai, China) at 25 °C (±2 °C). The working electrode was a modified GCE (2 mm in diameter). A saturated calomel electrode (SCE) served as the reference electrode, and a Pt wire served as the counter electrode. The amperometric response of the enzyme electrode to glucose was measured at an applied potential of 0.6 V (*vs.* SCE) under stirring. The supporting electrolyte was 0.1 M air-saturated PBS (pH 6.0). After the background current reached steady value, glucose solution was injected into the solution and the current was recorded.

3. Results and discussion

3.1. Characterization of Pt/CMK-3

The TEM image of the synthesized Pt/CMK-3 material is shown in Fig. 1. The CMK-3 displays well-ordered pore structure with pore size of about 4 nm. A large number of Pt nanoparticles are embedded in the pore channels. The Pt nanoparticles show narrow size distribution and the average diameter is about 2 nm. The Pt/CMK-3 material has large surface area, which is favorable to the immobilization of GOx. The high dispersion of Pt nanoparticles can make their electrocatalytic efficiency increase.

In addition, the electrochemical behavior of Pt/CMK-3 is also investigated. As can be seen in Fig. 2, the Pt/CMK-3-PVA/GCE shows sensitive response to H_2O_2, which is due to the electrochemical oxidation of H_2O_2 on the electrode surface at 0.6 V.

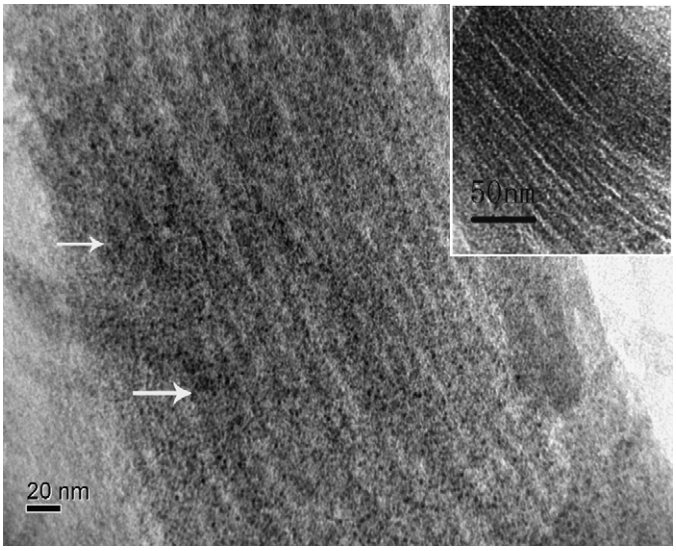

Fig. 1. TEM images of Pt/CMK-3 material taken along to the channel direction. Inset: TEM image with different magnification.

However, the current response caused by the CMK-3-PVA/GCE is quite small, which is only one-fifteenth as large as that of Pt/CMK-3-PVA/GCE. This indicates that the Pt nanoparticles deposited on mesoporous carbon have good catalytic activity to the oxidation of H_2O_2. The response to H_2O_2 covers a large concentration range, thus Pt/CMK-3 has great potential in constructing biosensor.

3.2. Amperometric responses of the enzyme electrodes to glucose

Fig. 3 shows the typical amperometric responses of different enzyme electrodes to glucose. The current response features step-like increase with successive addition of glucose, which is due to the enzyme catalytic oxidation of glucose. As for CMK-3-GOx-gelatin/GCE, the response current is rather small. Furthermore, the response decreases rapidly with glucose concentration rising. However, the Pt/CMK-3-GOx-gelatin/GCE displays sensitive and fast response to glucose, the response time ($t_{95\%}$) is less than 15 s. What is more, the response current is about 24 times as large as that of CMK-3-GOx-gelatin/GCE for 1 mM glucose. Hence, it can be proposed that Pt nanoparticles enhance the current response to glucose significantly. The response of glucose biosensor is related to the electrochemical oxidation of H_2O_2 produced in the enzymatic catalytic reaction. The reaction mechanism can be described as follows [4]:

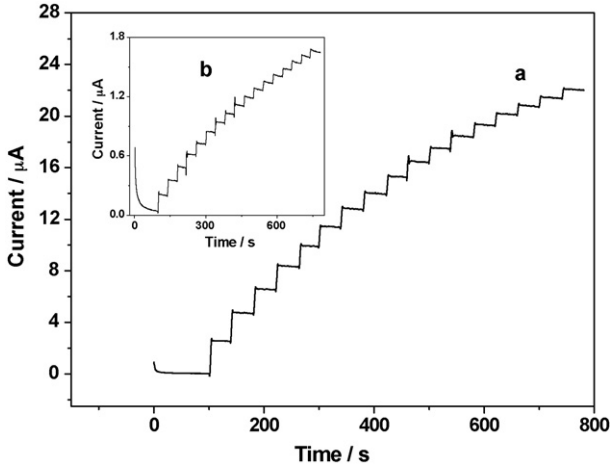

Fig. 2. Steady-state current response of Pt/CMK-3-PVA (a) and CMK-3-PVA (b) films coated GCEs to the addition of 0.975 mM H_2O_2 in the stirred PBS (pH 7.0). Applied potential: 0.6 V.

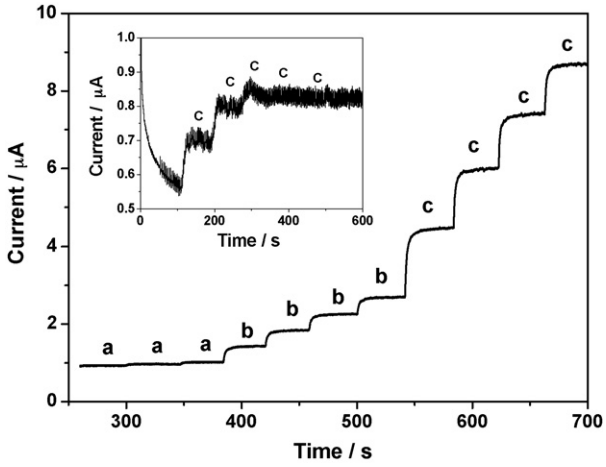

Fig. 3. Steady-state current response of Pt/CMK-3-GOx-gelatin/GCE to glucose in the stirred PBS (pH 6.0) at 0.6 V. Glucose concentrations: (a) 2×10^{-5} M, (b) 2×10^{-4} M, (c) 1×10^{-3} M; inset: the response of CMK-3-GOx-gelatin/GCE.

$$\text{Glucose} + \text{GOx}_{ox} \rightarrow \text{Gluconic acid} + \text{GOx}_{red} \quad (1)$$

$$\text{GOx}_{red} + O_2 \rightarrow \text{GOx}_{ox} + H_2O_2 \quad (2)$$

$$H_2O_2 \rightarrow O_2 + H_2O + 2e \quad (3)$$

In this case, the Pt nanoparticles deposited on CMK-3 can catalyze the oxidation of H_2O_2 markedly because they are highly dispersed and the loading amount is quite large. As a consequence, the Pt/CMK-3-GOx-gelatin/GCE shows sensitive current response to glucose.

3.3. Optimization of experimental conditions

3.3.1. Effects of fabrication parameters

The concentrations of GOx and gelatin influence current response significantly as shown in Fig. 4. When the concentration of Pt/CMK-3 is 5 mg ml^{-1}, with the concentration of GOx increasing, the response current increases until it reaches the maximum at 8 mg ml^{-1}, and then decreases slightly. When the concentration of GOx is high enough, the amount of GOx may exceed the maximum immobilization-amount of the Pt/CMK-3-gelatin matrix. Thus the response current stops increasing, even decreases. As for gelatin, it acts as binder. When gelatin concentration changes from 0.2 to 1 wt%, a maximum current response is observed at 0.6 wt%. When its concentration is too low or too high, GOx and Pt/CMK-3 cannot be immobilized effectively on the electrode surface or the current response becomes small due to the insulated property of gelatin. Accordingly, 8 mg ml^{-1}

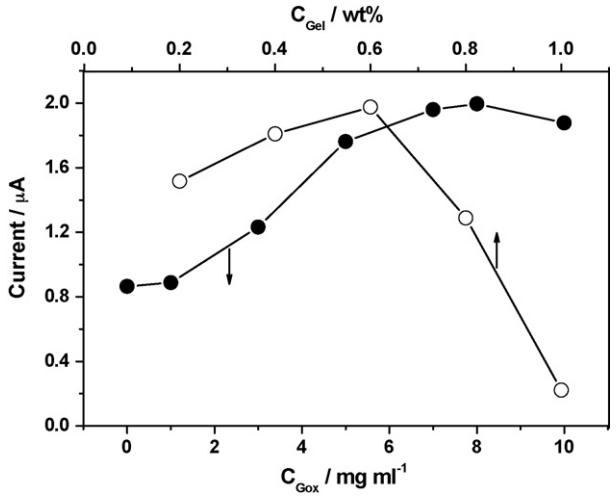

Fig. 4. Effects of the concentrations of GOx and gelatin on the response current of the Pt/CMK-3-GOx-gelatin/GCE. Steady-state currents are measured at 0.6 V in the stirred PBS (pH 6.0) with 1 mM glucose.

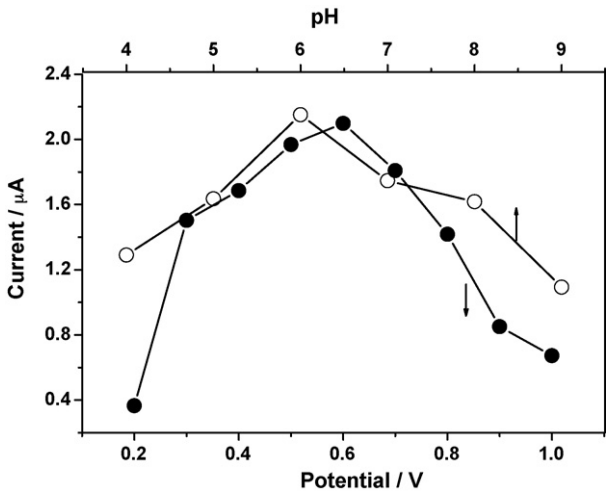

Fig. 5. Effects of the solution pH and applied potential on the response current of Pt/CMK-3-GOx-gelatin/GCE. Other conditions are the same as those in Fig. 4.

GOx and 0.6 wt% gelatin are chosen to fabricate the enzyme electrode.

To avoid the loss of GOx chemical cross-linking with glutaraldehyde is introduced. Without covalent cross-linking, the biosensor does not exhibit sharp step-like response and the current signal is small. With increasing cross-linking time, the response time decreases. But when it exceeds 10 min, the response time almost stops changing. Glutaraldehyde can react with amino groups of GOx and gelatin to generate the intramolecular or intermolecular covalent linkages [28], leading to the formation of a three-dimensional net structure. The reason for the improvement of current response after covalent cross-linking is probably related to the netlike structure as it benefits mass transfer. In addition, the three-dimensional net structure can improve the stability of the biosensor. When gelatin is replaced by PVA, no such effect is observed because PVA cannot react with glutaraldehyde.

3.3.2. *Effects of operation parameters*

The effects of applied potential and solution pH on the response current of Pt/CMK-3-GOx-gelatin/GCE are also studied (Fig. 5). The response current increases rapidly when the applied potential changes from 0 to 0.6 V. But when the potential is higher than 0.6 V, the response current falls down. This is related to the electrochemical oxidation of H_2O_2. The effect of potential on electrocatalytic oxidation of H_2O_2 at platinum electrodes was studied intensively by Hall et al. [29]. When a sufficiently positive potential is applied (e.g. +0.6 V *vs.* Ag/AgCl), the rate limiting reaction is: $Pt(OH)_2{\cdot}H_2O_2 \rightarrow Pt + 2H_2O + O_2$.The response current depends on the surface coverage of $Pt(OH)_2{\cdot}H_2O_2$, while $Pt(OH)_2{\cdot}O_2$ and $Pt(OH)_2{\cdot}H_2O_2{\cdot}H^+$ can inhibit this reaction. In this work, the Pt nanoparticles are imbedded in the pore channels; the diffusion of the O_2 is more difficult. At higher potential more O_2 is produced. These contribute to the formation of $Pt(OH)_2{\cdot}O_2$. In addition, the acidic condition (i.e. pH 6.0 PBS) benefits the formation of $Pt(OH)_2{\cdot}H_2O_2{\cdot}H^+$. When the two inhibiting species increase, the surface coverage of $Pt(OH)_2{\cdot}H_2O_2$ decreases. So the response current decreases when the potential is too higher.

The effect of solution pH on response current is studied between pH 4.0 and 9.0. The maximum response current is obtained at pH 6.0 (Fig. 5). Similar results were reported in literatures [6,11]. According to literature [30], good response for free enzyme occurred in the pH range of 4.0–7.0. Therefore, it can be concluded that the GOx immobilized in the Pt/CMK-3-gelatin matrix has higher bioactivity at about pH 6.0.

The effect of temperature on the response current is presented in Fig. 6. The response current increases when it changes from 2 to 52 °C, resulting from the increase of enzyme reaction rate. But the response current lowers when the temperature is over 52 °C because of the denaturation of GOx. The response current reaches the maximum at 52 °C. This temperature is higher than those observed for GOx entrapped in zeolite matrix [31] (i.e. 45 °C) and Pt nanoparticles/nano-fibrous [11] (i.e. 45 °C). It indicates that the Pt/CMK-3-GOx-gelatin/GCE can handle a

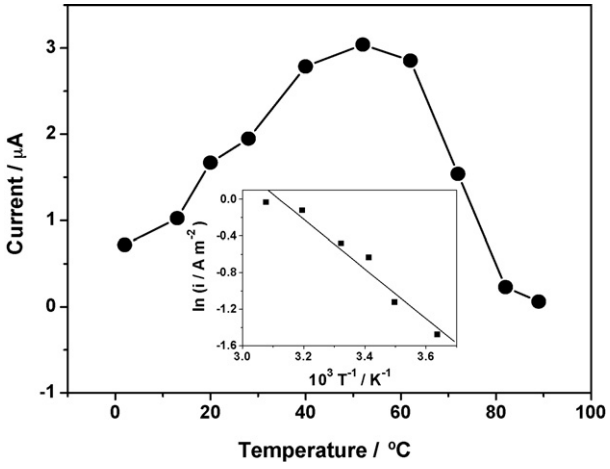

Fig. 6. Effect of temperature on the response current of Pt/CMK-3-GOx-gelatin/GCE. Inset: plot of the ln *i* *vs.* T^{-1}; other conditions are the same as those in Fig. 4.

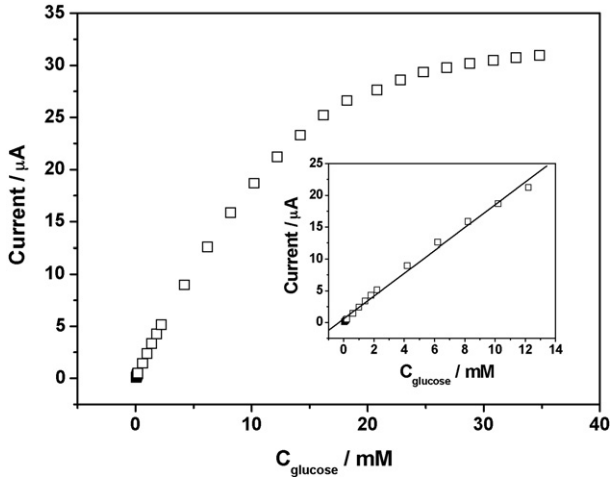

Fig. 7. Variation of response current with glucose concentration. Inset: the linear plot of response current *vs.* the concentration of glucose; other conditions are the same as those in Fig. 4.

wider temperature range, which can be attributed to the protection effect of mesoporous carbon matrix. The dependence of response current on temperature in the range of 2–52 °C can be expressed through the Arrhenius relationship [32,33]. The activation energy can be calculated according to the slope of the plot of logarithm of current density (ln *i*) *versus* reciprocal of temperature (T^{-1}) (Fig. 6). It is 22.54 kJ mol^{-1}, which is quite different from that reported for the free enzyme, namely 14.63 kJ mol^{-1} [34], but is in good accordance with those reported for immobilized GOx (i.e. 22.4–50 kJ mol^{-1}) [33].

3.4. Calibration curve, reproducibility and stability

Fig. 7 shows the calibration curve of glucose. The response current increases with the glucose concentration rising. When it is enough high, the current tends to stable, showing the characteristic of Michaelis–Menten kinetics. The response current is linear to the concentration of glucose over the range from 0.04 to 12.2 mM. The regression equation is $I = 5.53 \times 10^{-7} c_{\text{glucose}} + 1.79 \times 10^{-3}$ (A, M, $R = 0.996$, $n = 10$). The detection limit is 1 μM (S/N = 3). According to the Lineweaver–Burk equation [35], the apparent Michaelis–Menten constant (K_m^{app}) and the maximum current density (i_{max}) of the enzyme reaction at the Pt/CMK-3-GOx-gelatin/GCE can be calculated. In this case they are 10.8 mM and 908 μA cm^{-2}, respectively. These values are close to the reported values for free enzyme [6].

The reproducibility of the Pt/CMK-3-GOx-gelatin/GCE has been evaluated by comparing the response currents of 10 enzyme electrodes prepared. The relative standard deviation (RSD) was 8.1% when 1 mM glucose was determined. The operational stability was examined too. An enzyme electrode was used to determine 1 mM glucose for 10 times continuously, and the RSD of the response current was 4.2%. To test the long-time stability of the enzyme electrode under storage condition (pH 7.0 PBS, 4 °C), an enzyme electrode was used to determine 1 mM glucose solution one time everyday. Up to 30 days the response current retained 95.1% of the initial value. Compared with those glucose biosensors based on enzyme immobilized on Pt nanoparticles and carbon nanotubes matrixes [6,10], the Pt/CMK-3-GOx-gelatin/GCE has better storage stability. This indicates that the mesoporous carbon-gelatin film after covalent cross-linking is very stable and the matrix can prevent enzyme from leaking, contamination and biodegradation.

Table 1
The determination of glucose levels in serum samples

Samples	Provided by the local hospital (mM)	Determined by current method (mM)	Relative error (%)
1	4.95	4.80	−3.03
2	5.57	5.53	−0.72
3	4.51	4.73	4.88
4	4.44	4.76	7.21

3.5. Interference and serum sample analysis

The interference of some electroactive compounds to the glucose response was examined. When the glucose concentration was 5.6 mM, the deviation of response was calculated according to $(I_i - I_g)/I_g$, where I_i and I_g were the steady-state current recorded for solutions containing glucose and interferent or glucose alone. At Pt/CMK-3-GOx-gelatin/GCE, the deviations caused by ascorbic acid (0.1 mM), *p*-acetaminophenol (0.05 mM) and uric acid (0.5 mM) were 0.44%, 4.69% and 13.2%, respectively. This indicates that the biosensor suffers some influence from the coexistents in serum samples. Therefore, 1 μl 1 wt% Nafion was cast on the surface of Pt/CMK-3-GOx-gelatin/GCE to prevent the interference. And then, at Nafion/Pt/CMK-3-GOx-gelatin/GCE, the deviations were in the acceptable range (±10%). The current response of the resulting biosensor was linear to the concentration of glucose from 0.02 to 16.9 mM ($R = 0.993$). However, for 1 mM glucose the response current decreased by 21% in comparison with that of Pt/CMK-3-GOx-gelatin/GCE. The detection range is sufficient for monitoring glucose level of human blood, which is generally 4.4–6.6 mM [36].

The determination of glucose in human serum samples was performed with the Nafion/Pt/CMK-3-GOx-gelatin/GCE. The serum samples were assayed by the glucose oxidase method on a Hitachi 7080 biochemical automatic analyzer in local hospital at the same time. For current method, 50 μl serum was added into the stirred 5 ml PBS (pH 6.0), and amperometric response was recorded at 0.6 V. The results were shown in Table 1. The recoveries for the electrochemical assays of 1–3 mM glucose were between 94.4% and 98.3%. The glucose levels determined in this work were close to the values declared by hospital, indicating that the fabricated glucose biosensor has practical potential.

4. Conclusions

A glucose biosensor has been fabricated based on GOx immobilized in Pt/CMK-3 matrix. Pt nanoparticles loaded have strong electrocatalysis to the oxidation of H_2O_2. The glucose biosensor shows sensitive and rapid response to glucose. With an extra

Nafion film to eliminate the interferents, the resulting biosensor could be used to determine the glucose levels of serum samples. Owing to the protection effect of mesoporous material to GOx, the biosensor has high thermal stability and long-time stability. Therefore, the Pt nanoparticles/mesoporous carbon CMK-3 system is promising in biosensor construction.

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