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Highly Stable Hydrogen Peroxide Biosensor Based on Gelatin-Hierarchical Porous Carbon Obtained from Fish Scales Modified Glassy Carbon Electrode

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Biosensors for continuous application are becoming more important, and long-life enzyme electrodes have not been commercialized due to the lack of stability of its components. A highly stable biosensor for hydrogen peroxide (H_2O_2) based on gelatin-hierarchical porous carbon obtained from fish scales has been developed in this work. Catalase (Cat) immobilized on gelatin-hierarchical porous carbon was realized by the adsorption-crosslinked, here glutaraldehyde (GAD) was used as cross-linking agent. The surface morphologies of modified glass carbon electrodes(GCE) were characterized by scanning electron microscopy (SEM), and the electrochemical behaviors of the biosensor were characterized by cyclic voltammogram (CV) and electrochemical impedance spectroscopy (EIS). This biosensor exhibited a linear range from 0.2 to 10.7 mM with a correlation coefficient (R^2) of 0.999, a low detection limit of 0.7 μM and a sensitivity of 74.69 $\mu AmM^{-1}cm^{-2}$. Especially, the stability of the as-prepared biosensor was satisfied at room temperature, which means there was almost no change in the performance even after 100 continuous scans, and the storage stability is over 30 days with retention of 98.7% activity only.

Keywords: Gelatin, Porous carbon, Operational and long-term stability, Catalase, Hydrogen peroxide biosensor

1. INTRODUCTION

Hydrogen peroxide (H_2O_2) is usually used to as disinfectant, fungicide, bleaching agent and reductant in many fields including food industry, pharmaceutical industry, printing and dyeing industry,

and chemical industry [1-4]. H_2O_2 is harmful for biological systems and appears to be involved in the neuropathology of central nervous system diseases [5]. So the detection of H_2O_2 becomes more and more important in the food analyses, pharmaceutical, biological, industrial settings, clinical control and environment protection [6]. Up to now, several methods such as chemiluminescence, spectral techniques and electrochemical approaches have been used for the assay of H_2O_2 [7]. Among these, electrochemical approaches based on amperometric enzyme biosensors have received considerable interest because of their convenience, fast response time, high reliability, sensitivity and low cost. In the area of biosensor, some of the major obstacles in the systems success are the low sensitivity and stability. In recent years, tens of thousands of papers about how to increase the sensitivity levels of the biosensors have been published, but how to enhance the stability has been a little reported. It is well known that the good operational stability of biosensor is premise of accurate measurement, and its long-term lifetime is not only beneficial for biosensor transport and storage but also helps decrease per measurement costs [8]. Nowadays, in order to realize the commercialization of the biosensor, many researchers focused on improving its operational stability and long-term stability. For many biosensors, poor stability is mainly caused by electrode fouling, enzyme inactivation and desorption from immobilization materials, and the stability of the enzyme layer limits the application lifetime of the sensor [9-10].

Research efforts are mainly focused on the stabilization of enzymes in porous carbon materials with pores or internal spaces. Porous carbon materials are more exciting candidates for enzyme immobilization compared with conventional materials due to their large surface area and opened pore structure that allows high enzyme loadings. We reported here a hierarchical porous carbon material (HPC) which was prepared with fish scale using a natural template, and it has been confirmed as an excellent electrode material [11-12]. HPC has been employed to immobilize enzyme, thereby designing and preparing a novel hydrogen peroxide biosensor. It is well-known, fish scale is composed of an organic component, which is mostly collagen fibers, and inorganic calcium-deficient hydroxyapatite, which is well-dispersed in the organic component following a specific manner. So, the organic component provides the carbon for porous carbon materials. Meanwhile the mineral phase acts as a natural template for the formation of a hierarchical porous structure because it can be easily eliminated after the preparation process [13]. This hierarchical porous carbon material possesses high surface area and well developed pore structure, which could provide a large amount of adsorption site [14-16]. The hierarchical porous structure can also provide channels for ions transfer, reduce the resistance of ions transfer, and result in the rapid transfer of ions [17-19]. Gelatin (Gel) has been selected as the multifunctional supporting material in the biosensor for catalase (Cat) immobilization [20-25] considered it has satisfied property for dispersing the electrode materials, high adhesion ability for bonding different types of small particles, excellent biocompatibility for maintaining high activity of enzyme.

In this study, Cat was immobilized in a novel matrix of Gel-HPC. The immobilization of Cat was achieved by the physical adsorption-chemical crosslinked method. Thus there was reduced the loss of enzyme and ensured the stability of biosensor.

2. EXPERIMENTAL

2.1. Reagents and materials

HPC was prepared according to the method described in a previous study [11]. Gelatin was obtained from animal bones (type B). Catalase (Cat, EC 1.11.1.6, 3500 units/mg) was purchased from Aladdin Reagent Inc. 30% H_2O_2 were obtained from Beijing Chemical Works. Phosphate buffer solution (PBS, 0.1 M, pH 7) was prepared by mixing the stock standard solutions of 0.1 M Na_2HPO_4 and 0.1 M NaH_2PO_4. Double-distilled water was used for the preparation of all aqueous solutions, and all other chemicals were of analytical grade and used without further purification.

2.2. Apparatus and measurements

All electrochemical experiments were performed with a CHI 660C electrochemical workstation (CH Instruments, Shanghai, China). A conventional three-electrode system using 3 mm diameter modified and unmodified glass carbon electrode (GCE) as working electrode, a platinum wire as counter electrode, and a saturated calomel electrode (SCE) as reference electrode. All measurements were carried out at room temperature. Cyclic voltammograms (CVs) were recorded in 0.1 M PBS (pH 7) from 0.6 V to -1.0 V at a scan rate of 50 mV/s. Electrochemical impedance spectroscopy (EIS) was carried out in pH 7 PBS containing 5 mM $K_3Fe(CN)_6$ and 0.1 M KCl with the frequencies ranging from 1 Hz to 10^5 Hz. For amperometric i-t measurement, -0.4 V was applied as an operating potential. After initial current stabilization, small aliquots of a H_2O_2 stock solution (0.1 M in 0.1 M PBS, pH 7) were successively added at a time interval of 50 s.

2.3. Preparation of enzyme electrode

GCE surface was polished with 0.05 μm alumina powder on a polishing cloth, then washed several times with double distilled water, and dried at room temperature.

Dropping method was employed to fabricate the enzyme electrode, a detail procedure was as following: a mixture solution was prepared by adding 2 mg HPC and acetylene black (AB, Jinpu. Corp., China) in 1mL 1% gelatin aqueous solution and was ultrasonicated for 0.5 h. 10 mg/mL Cat solution was prepared in 0.1M pH 7 PBS and stored at 4℃. Subsequently, the pretreated GCE was modified by dropping 10 μL of the Gel-HPC solution and allowed to be dried in ambient air at room temperature for 1 h; the obtained electrode was coated with 10 μL Cat solution, which was incubated at room temperature for 1 h; then 10 μL of 0.25% Glutaraldehyde (GAD) solution was layered on top of the Gel-HPC/Cat modified GCE and dried; after evaporation of water, the modified electrode was washed with PBS to remove the unbound Cat and the resulted Gel-HPC/Cat/GAD modified GCE was stored at room temperature.

3. RESULTS AND DISCUSSION

3.1. Morphological characterization of modified electrode

The surface morphologie of modified GCE were visualized using SEM. Figure 1 presents the typical SEM images of (A) HPC and (B) Gel-HPC/Cat/GAD modified GCE surface. The SEM image of the HPC shows a hierarchical porous structure, and the developed porous structure is favorable for the enzyme immobilization and provides channels for ions transfer. It is also clearly seen that the Gel-HPC/Cat/GAD film exhibited a uniform porous structure, and porous structure of the composite film is more conducive to the rapid transfer of electrons and ions.

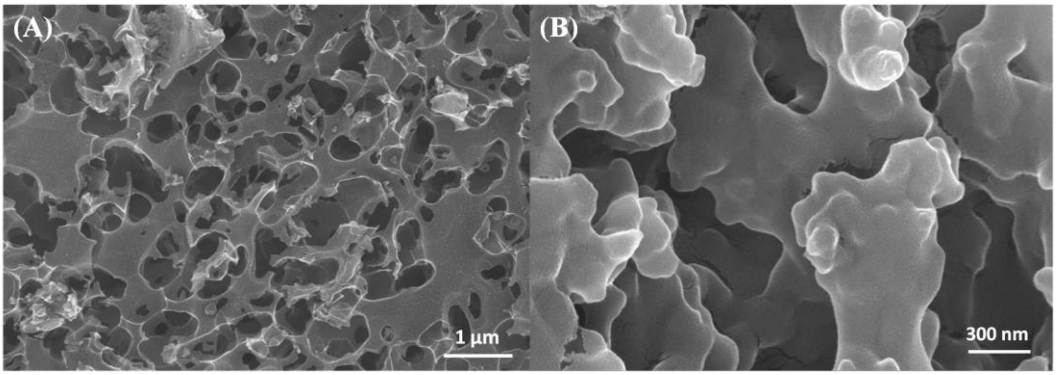

Figure 1. SEM images of HPC (A) and Gel-HPC/Cat/GAD film (B).

3.2. Direct electrochemistry of calatase

The cyclic voltammograms (CVs) of different modified electrodes were shown in Figure 2. Figure 2A compared the cyclic voltammograms of (a) bare GCE, (b) Gel, (c) Cat/GAD, (d) Gel-HPC and (e) Gel-HPC/Cat/GAD films modified GCEs in the absence of H_2O_2. Gel-HPC and Gel-HPC/Cat/GAD films modified GCEs in PBS have the similar response, and the electrochemistry signals are significantly enhanced, compared with the bare, Gel and Cat/GAD electrodes. It clearly confirmed the important electrochemical role of HPC in the electronic transmission and response current amplification process of modified electrodes. As shown in Figure 2B, no peak was observed at (a) bare GCE, (b) Gel and (d) Gel-HPC films modified electrodes and thus Gel and HPC are electroinactive within the potential windows. For (c) Cat/GAD and (e) Gel-HPC/Cat/GAD, as soon as H_2O_2 was added in electrolyte solution, compared with the system with no H_2O_2 present, an obvious increase in the reduction peak was observed. Cat in the Gel-HPC networks retains its bioactivity and catalyzes the reduction of H_2O_2. The generic reaction of the immobilized Cat to H_2O_2 reduction can be explained by the following scheme [26]:

$$\text{Cat-Fe(III)} + H_2O_2 \rightarrow \text{Cat-Fe(IV)=O} + H_2O$$

$$\text{Cat-Fe(IV)=O} + H_2O_2 \rightarrow \text{Cat-Fe(III)} + H_2O + O_2$$

The cyclic voltammogram for Cat/GAD shows poorly defined peaks while that for Gel-HPC/Cat/GAD shows a significant cathode reduction peak, which are attributed to the direct electron

transfer (DET) between the electroactive center of the immobilized Cat and the electrode surface. As can be seen in Figure 2B (e), the redox process is an almost reversible electrochemical process. The significant cathode reduction peak is located at about -0.4 V [27-28].

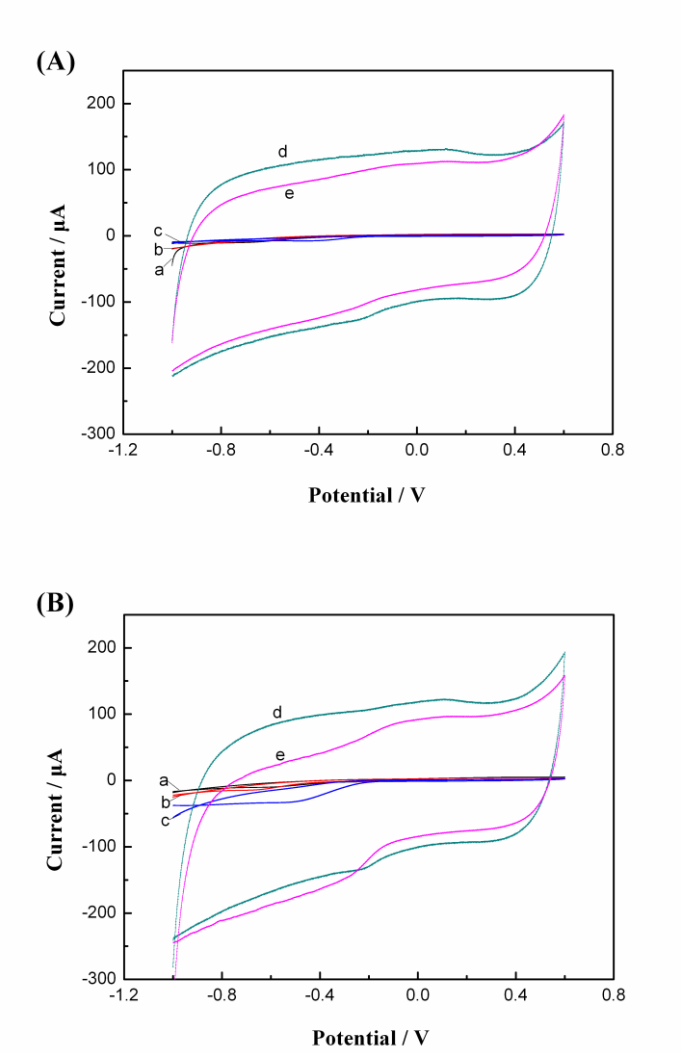

Figure 2. CVs of (a) bare GCE, (b) Gel, (c) Cat/GAD, (d) Gel-HPC and (e) Gel-HPC/Cat/GAD films modified GCEs in pH 7 PBS at the scan rate of 50 mV/s in the (A) absence and (B) presence of H_2O_2.

The influence of scan rate on the electrochemical response of Gel-HPCs/Cat/GAD modified GCE was further investigated. As displayed in Figure 3A, well-defined quasi-reversible redox peaks were observed at different scan rates. The peak current increased while the peak potential shifted slightly with the increase of the scan rate. The peak current exhibited a linear dependence (R^2= 0.9993) on the scan rates ranging from 10 to 100 mV/s (as shown in Figure 3B). The results manifested a typical surface-controlled electrochemical behavior, and Gel-HPC matrix can effectively promote the electron transfer process between the Cat and the electrode surface [29-31].

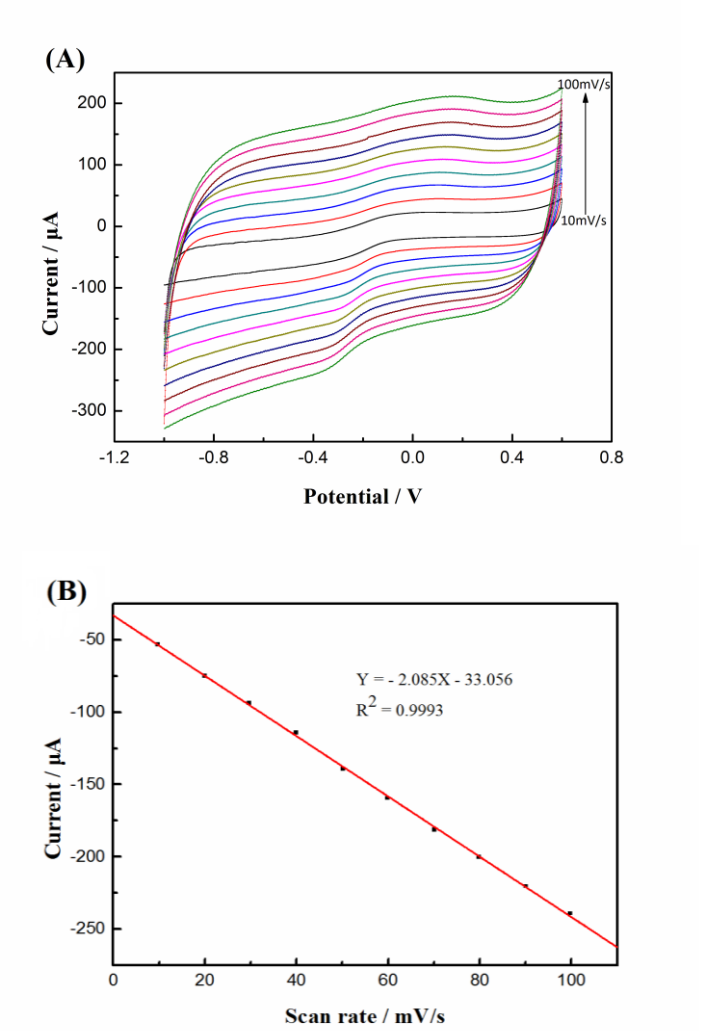

Figure 3. (A) Influence of scan rate on electrochemical responses of Gel-HPCs/Cat/GAD modified GCE in pH 7 PBS with scan rates from 10 mV/s to 100 mV/s, respectively. (B) Linear relationship of reduction peak current on different scan rates from 10 mV/s to 100 mV/s.

3.3. Electrochemical impedance spectroscopy (EIS)

EIS was an effective tool for studying the interface properties of surface-modified electrodes [32-33]. In EIS, the semicircle portion observed at high frequencies in the Nyquist diagrams corresponds to the electron transfer limiting process [34]. Figure 4 showed the EIS of (a) Gel, (b) Cat/GAD, (c) Gel-HPC and (d) Gel-HPC/Cat/GAD films modified GCEs in pH 7 PBS containing 5 mM $K_3Fe(CN)_6$ and 0.1 M KCl across the frequency range from 1 Hz to 10^5 Hz. Randles equivalent circuit model (inset in Figure 4) has been used to fit the experimental data where, R_s is the ohmic resistance of the electrolyte, R_{et} is the charge transfer resistance, C_{dl} is the double layer capacitance and Z_w is the Warburg impedance [35]. As can be seen from Figure 4, EIS of the Gel and Cat/GAD showed an enlarged semicircle than that of other films modified GCEs, owing to the insulating property of Gel, Cat and GAD. On the other hand, Gel-HPCs and Gel-HPC/Cat/GAD exhibited small semicircle, and the values of R_{et} were 77.2 Ω and 52.8 Ω, respectively, revealing that HPC could act as a good electron-transfer interface between the Cat and the electrode. Gel-HPC/Cat/GAD modified GCE

exhibited much smaller semicircle than Gel-HPCs, owing to the uniform porous structure of Gel-HPC/Cat/GAD film. This structure can reduce the resistance of electron transfer, and result in the rapid transfer of electrons.

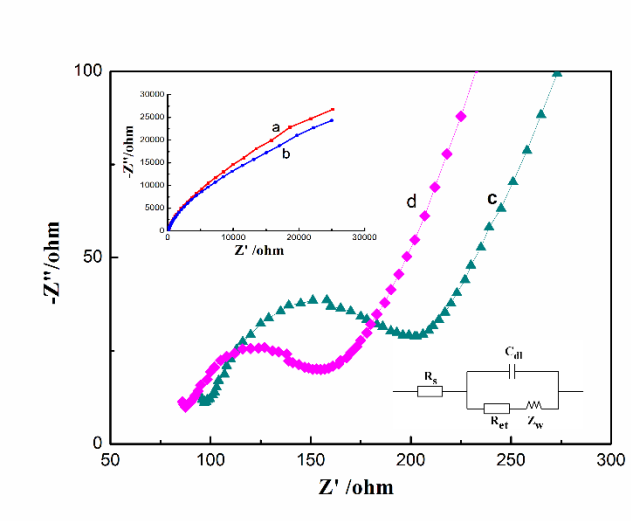

Figure 4. EIS of (a) Gel, (b) Cat/GAD, (c) Gel-HPCs and (d) Gel-HPC/Cat/GAD films modified GCEs in pH 7 PBS containing 5 mM $K_3Fe(CN)_6$ and 0.1 M KCl across the frequency range from 1 Hz to 10^5 Hz. Inset: randles equivalent circuit model.

3.4. Amperometric performance of the biosensor

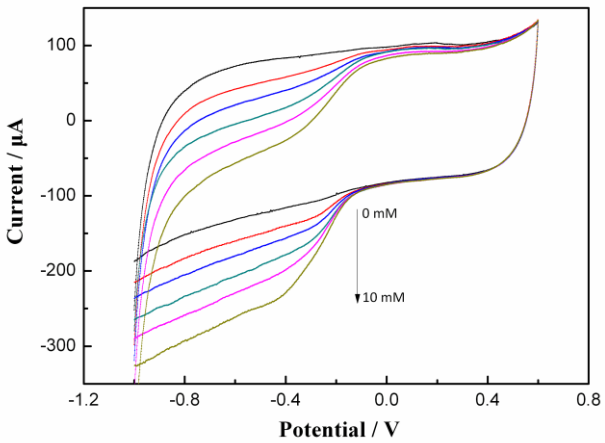

Figure 5. CVs of Gel-HPC/Cat/GAD film modified GCE in pH 7 PBS containing 0, 2, 4, 6, 8 and 10 mM H_2O_2 at the scan rate of 50 mV/s.

Catalase catalyzes the decomposition of hydrogen peroxide to water and oxygen. Cat-based electrochemical techniques are therefore finding potential applications for the detection of H_2O_2 in the field of environment, chemistry and biology. The as prepared Gel-HPC/Cat/GAD film was further evaluated in H_2O_2 sensing using the direct electrochemistry of Cat. Figure 5 showed CVs of Gel-HPC/Cat/GAD film modified electrode in the absence and presence of different concentrations of

H_2O_2 in pH 7 PBS. There is a notable enhancement of cathode reduction peak currents along with a decrease in the anodic oxidation peak currents with successive additions of H_2O_2, showing electrocatalytic reduction process of H_2O_2.

The typical amperometric responses of Gel-HPC/Cat/GAD film modified electrode at an applied electrode potential of -0.4 V toward H_2O_2 was shown in Figure 6A. When H_2O_2 was added into the stirring buffer solution, the response current raised quickly and steeply to a stable value, and the electrode achieved the steady state current within 3-5 s. The amperometric response current increased linearly with H_2O_2 concentrations between 0.2 mM and 10.7 mM with a coefficient of determination (R^2) of 0.999, evident from the calibration plot (as shown in Figure 6B). The electrode has a sensitivity of 74.69 $\mu AmM^{-1}cm^{-2}$ and a detection limit 0.7 μM with a signal-to-noise ratio of 3. The fast response and high sensitivity may be due to the hierarchical porous structure of HPC and the facile diffusion of H_2O_2 in the uniform porous structure of Gel-HPC/Cat/GAD film.

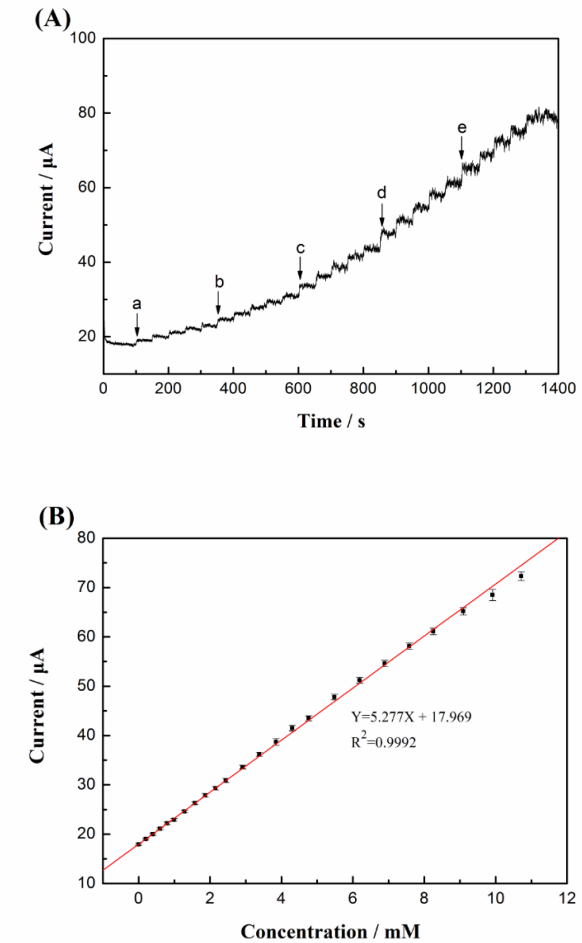

Figure 6. (A) The amperometric response of Gel-HPC/Cat/GAD film modified GCE at -0.4 V upon successive additions of H_2O_2 in 0.1 M pH 7 PBS. (B) Amperometric response curve for H_2O_2.

3.5. Interference study of the biosensor

One of the most important analytical factors for an amperometric biosensor is the selectivity of the biosensor to target analyte. In this study, ascorbic acid (AA), uric acid (UA) and dopamine (DA)

were used as endogenous interfering substances [36-38]. The biosensor showed no response to 0.3 mM of these compounds (as shown in Figure 7), which means the studied interference materials did not influence the determination of H_2O_2 at corresponding concentrations, and provides the biosensor with high selectivity.

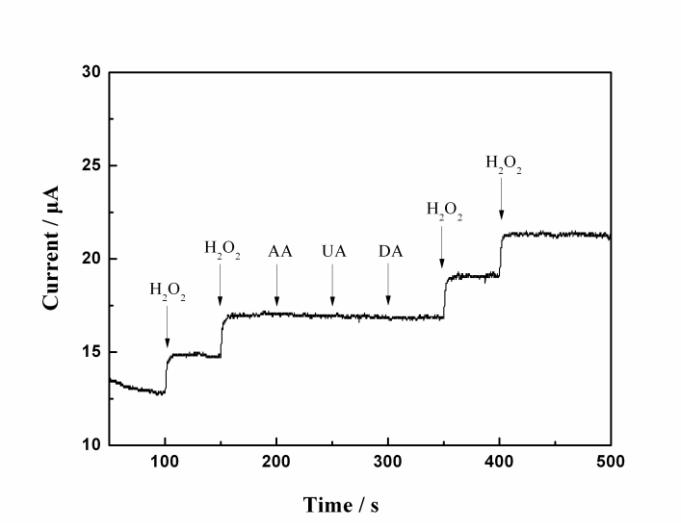

Figure 7. The amperometric response of Gel-HPC/Cat/GAD film modified GCE in 0.1 M pH 7 PBS containing 0.3 mM H_2O_2, spiked with 0.3 mM AA, UA, DA.

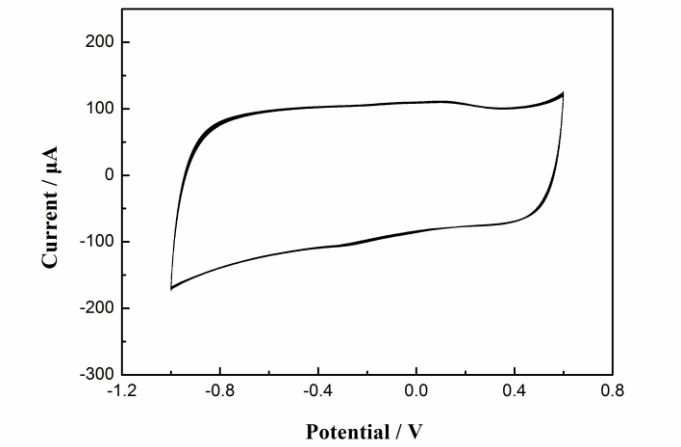

Figure 8. CVs of Gel-HPC/Cat/GAD film modified GCE in pH 7 PBS at the scan rate of 50 mV/s for 100 cycles.

3.6. Stability of the biosensor

A continuous measurement of CVs had been carried out in 0.1M pH 7 PBS at room temperature to evaluate the operational stability of this biosensor. As shown in Figure 8, it was found that peak current for H_2O_2 basically coincide with its initial value and no obvious potential shift was

observed after 100 scans, which showed that the modified electrode had a good operational stability. Therefore, continuous monitoring can be achieved. Long-term storage stability of biosensors is one of the most important factors in case of their commercial use. The long-term stability of Gel-HPC/Cat/GAD film modified GCE prepared under optimum conditions was investigated. The modified electrode was washed with double distilled water, dried and stored in a dry condition at room temperature when not being in use. During one month, the modified electrode was tested every 2 days for H_2O_2, and there was no apparent decrease in the current response in the continuous 2 weeks' measurements (as shown in Figure 9). After that, the current responses gradually decreased. However, the current values still remained 98.7% of its initial response for H_2O_2. The biosensor in this work shows a higher stability than reported of HRP-$CoFe_2O_4$-ChIT/GCE biosensor (50 days, 4 ℃, 50%) [39], Cat/PLL/f-MWCNT biosensor (1 month, 4 ℃, 89%) [41], Pd core-Pt NDs-rGO sensor (2 weeks, 4 ℃, 93%) [45]. We have summarized various H_2O_2 sensors with the performances of detection limit, linear range, sensitivity and stability in Table 1. It is obvious that the proposed Gel-HPC/Cat/GAD film modified GCE exhibits the highest stability among all the H_2O_2 sensors listed. The excellent performance could be ascribed to the outstanding properties of the Gel-HPC/Cat/GAD film. Gelatin possessed the satisfied dispersion, high adhesion ability, and excellent biocompatibility which can provide a suitable environment for the enzyme to maintain high activity and improve the stability of biosensor. In addition, GAD cross-linking can effectively prevent the shedding of the enzyme which can also enhance the stability of biosensor. Given the aforementioned advantages, the biosensor was reliable for the detection of H_2O_2.

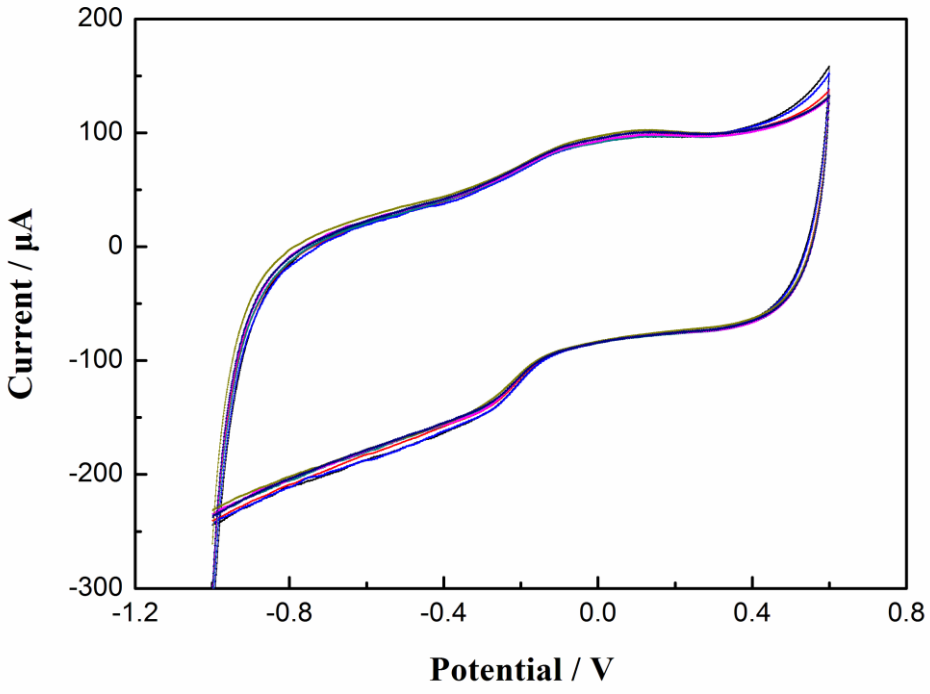

Figure 9. The storage stability of the Gel-HPC/Cat/GAD film modified GCE (CVs of the electrode in pH 7 PBS containing 4 mM H_2O_2 at the scan rate of 50 mV/s for 2 consecutive weeks).

Table 1. Comparison of performances of different electrochemical biosensors for determination of H_2O_2.

Biosensor	Detection limit (μM)	Linear range (mM)	Sensitivity ($\mu AmM^{-1}cm^{-2}$)	Stability	Reference
HRP-$CoFe_2O_4$-ChIT/GCE	2	0.03 – 8	0.023	50 days (4 ℃, 50%)	[39]
PAN-PNMThH HRP	3.2	0.005 – 60	35	30 days (4 ℃, 86.6%)	[40]
Cat/PLL/f-MWCNT	0.008	0.001 - 3.6	392	1 month (4 ℃, 89%)	[41]
CHIT/HRP/KNs/Au	12	0.04 – 6	750	3 weeks (4 ℃, 90%)	[42]
PEDOT/PB	0.16	0.5 – 839	-	30 days (room temperature, 90.8%)	[43]
Cat/MgO NPs/CPE	-	0.05 - 0.19	-	50 days (3 ℃, 92%)	[44]
Pd core-Pt NDs-rGO	0.027	0.005 – 0.5	672.753	2 weeks (4 ℃, 93%)	[45]
Mn_2O_3/Nf/GCE	0.07	0.0001 – 0.1265	-	30 days (room temperature, 93%)	[46]
NS-G/GCE	0.2	0.1 - 16.6	-	14 days (4 ℃, 94.1%)	[47]
Gel-HPC/Cat/GAD	0.7	0.2 - 10.7	74.69	1 month (room temperature, 98.7%)	this work

4. CONCLUSION

In the present work, a hydrogen peroxide biosensor with high stability was developed. A novel composite film that consists of hierarchical porous structure of HPC, a biomacromolecule Gel and GAD was successfully used to immobilize Cat. The fabricated hydrogen peroxide biosensor showed a good linear range, low detection limit, high sensitivity and good stability for the detection of H_2O_2 due to the role of HPC and Gel that formed the supporting materials. The effect modified by HPC do not only increase the fixed amount of Cat while maintaining biological activity of Cat, but also provide channels for ions transfer and reduce the resistance of ions transfer. Here, Gel was realized as an excellent adhesive, dispersing agent of HPC and carrier of multiple functions of Cat.

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