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Ordered mesoporous polyaniline film as a new matrix for enzyme immobilization and biosensor construction

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Abstract

Ordered mesoporous polyaniline film has been fabricated by electrodepositing from the hexagonal lyotropic liquid crystalline (LCC). Horseradish peroxidase (HRP), as a symbol biomolecule, was successfully immobilized on the film to construct a new kind of hydrogen peroxide biosensor. The biosensor combined the advantages of the good conductivity of polyaniline and the higher surface area of the ordered mesoporous film. Polyaniline could be served as a wire to relay electron between HRP and the electrode. The high surface area of the film supplied more sites for HRP immobilization, therefore increased the catalytic activity of the biosensor. The ordered mesoporous character of the film increased the rate of mass transport, which resulted in the improvement of sensor response and linearity. The biosensor displayed excellent electrocatalytic response to the detection of H_2O_2 in a concentration range from 1.0 μM to 2.0 mM with a detection limit of 0.63 μM. Good reproducibility, stability, high precision, wide linearity and low detection limit were assessed for the biosensor.

Keywords: Mesoporous; Ordered; Lyotropic liquid crystalline; Biosensor

1. Introduction

Conductive polymers have been widely used for the immobilization of biomolecules and the fabrication of sensors, because they have many attractive properties such as good biocompatibility and certain electrical conductivity [1–4]. In most cases, biomolecules were immobilized during the electrochemical polymerization process or after the films were activated with glutaraldehyde [5]. However, some polymers such as polyaniline are usually polymerized under strongly acidic conditions, which can have detrimental effects on the catalytic activity of most biomolecules, while glutaraldehyde contains complicated chemical species of documented cytotoxic nature [5]. Therefore, it is necessary using some new methods to overcome these shortcomings.

Porous materials with high surface areas have been applied for the immobilization of biomolecules [6–9]. The electrostatic interaction between the porous materials and biomolecules could minimize the possibility for the denaturation of the enzymes [10]. Several procedures have been employed to produce porous materials. Attard reported that porous films [11,12] with well-defined and regular nanostructures could be obtained by using the homogeneous nonionic lyotropic liquid crystalline (LCC) phases as templates. The resulted films were stable and contained ordered arrays of uniform pores extended over a hexagonal lattice.

Polyaniline (PANI), as a conductive polymer with unique conduction ability and high environmental stability, has been widely used [13,14]. Though micro- and nanostructured PANI have been prepared by chemical or electrochemical methods [15–18], so far few publications reported the preparation of ordered PANI films with mesoporous morphology and their application in biomolecule immobilization and biosensor construction.

In this communication, we presented the preparation and application of the nanostructured mesoporous PANI film using LCC as a template via a one-step electrochemical process. Scheme 1 shows the formation process of the ordered mesoporous PANI film from the hexagonal lyotropic liquid crystalline (LCC) phase and its application for HRP immobilization. The electrochemical procedure could offer more freedom in changing deposition conditions, resulting in easily adjusted pore size

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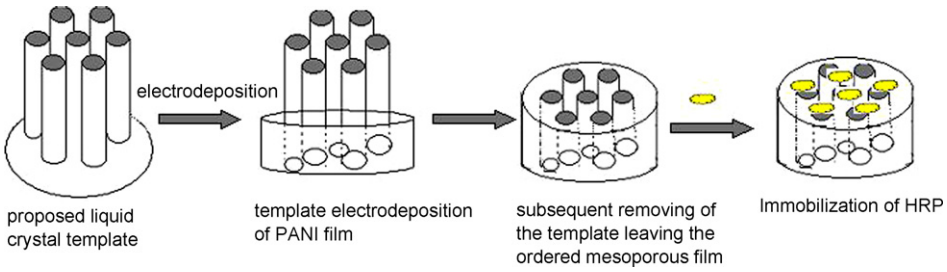

Scheme 1. The formation process of the ordered mesoporous PANI film from the hexagonal lyotropic liquid crystalline (LCC) phase and its application for HRP immobilization.

and controlled film thickness and properties [19]. The obtained PANI film combined the advantages of biocompatibility, high porosities and surface areas, easy biomolecule immobilization and electron-relaying ability. Electrochemical characterization for the mesoporous film was performed. Horseradish peroxidase (HRP), as a model biomolecule, has been successfully immobilized onto the mesoporous PANI film and its electrocatalytic properties were studied.

2. Experimental

2.1. Reagents

HRP (MW 44,000, EC 1.11.1.7) was obtained from Sino-American Biotechnology Co. Ltd. (Henan, China). The nonionic surfactant octaethyleneglycol monohexadecyl ether ($C_{16}EO_8$)] (Brij 56, 98%) was obtained from Fluka and used as received. Aniline was purchased from Shanghai Chemical Reagent Company (Shanghai, China). A 30% hydrogen peroxide (H_2O_2) solution was purchased from Beijing Chemical Reagent (Beijing, China). All other chemicals were of analytical grade and were used as received without any purification process. Deionized double-distilled water was used for making all the solutions (18.6 MΩ) (Millipore Co. Ltd.).

2.2. Apparatus and measurements

All electrochemical experiments were performed on a CHI 660a electrochemical analyzer (Shanghai Chenghua, China), using a conventional three-electrode system with a modified glassy carbon electrode (GCE) (3 mm in diameter, Shanghai Chenghua, China) as the working electrode, a platinum foil as the auxiliary electrode, and a saturated calomel electrode (SCE) as the reference electrode.

Scanning electron micrographs (SEM) of nonporous PANI and mesoporous PANI were taken on a LEO-1530VP field-emission scanning electron microscope. X-ray diffraction (XRD) was carried out using a Philips X'Pert Pr X-ray diffractometer with graphite monochromatized Cu Kα radiation with a 2θ range of 1–10°.

2.3. Preparation of mesoporous PANI film modified electrode

The template media used in the electrodeposition of mesoporous PANI film was binary systems comprised of Brij 56 and 0.1 M aniline in 1 M H_2SO_4 aqueous solution (46%, w/w). In the preparing of the mixtures, the surfactant was heated to ∼60 °C (above its melting point) in a glass vial and mixed manually using a glass rod on addition of the aqueous aniline solution. Mixing times of ∼10 min were required to obtain a homogeneous mixture. At room temperature, the mixture has a characteristic optical texture of hexagon liquid crystalline phase when viewed under a DMLP (Leica, Germany) polarizing light microscope equipped with a Linkam THMSE-600 heating stage and a temperature control unit.

Electrodeposition of the mesoporous PANI film from the liquid crystalline plating mixture onto the glassy carbon electrode was conducted by scanning the potential of the electrode between −0.2 and +0.8 V versus SCE at 50 mV s^{-1} at 25 °C. After deposition, the electrode was rinsed with deionized water to remove the surfactant. As a comparison, the nonporous PANI film was obtained by the same method except that the deposition solution used was 0.1 M aniline in 1 M H_2SO_4 aqueous solution in the absence of Brij 56.

For HRP immobilization, the resulting electrode was dipped into HRP solution (4.0 mg mL^{-1}, pH 7.0) overnight. Then the modified electrode was immersed into 0.1 M PBS 7.0 to wash off the loosely adsorbed HRP, and was stored at 4 °C in a refrigerator under dry conditions when not in use.

3. Results and discussion

The surface features of the PANI films deposited in the presence and absence of Brij 56 were compared by the scanning electron micrographs. The results were shown in Fig. 1A–C, respectively. The presence of Brij 56 significantly altered the morphology of the PANI film from the microsized particles (Fig. 1A) to the porous fibers (Fig. 1B and C). The nanofibers were formed as PANI grew along the long Brij 56 chains and interconnected into three-dimensional network. Because the porous structure of the nanofibers can contribute to the high surface area, the high coverage immobilization for biomolecules and the fast response for the constructed sensors can be obtained [20,21].

Low-angle X-ray diffraction spectrum confirmed the formation of the ordered mesoporous structure of PANI. In Fig. 2, four well-resolved peaks were observed, suggesting that a highly ordered, hexagonally arranged pore system was constructed. According to the Bragg equation [22], the strong, sharp peak could be indexed to the (0 0 1) diffraction of the mesoporous

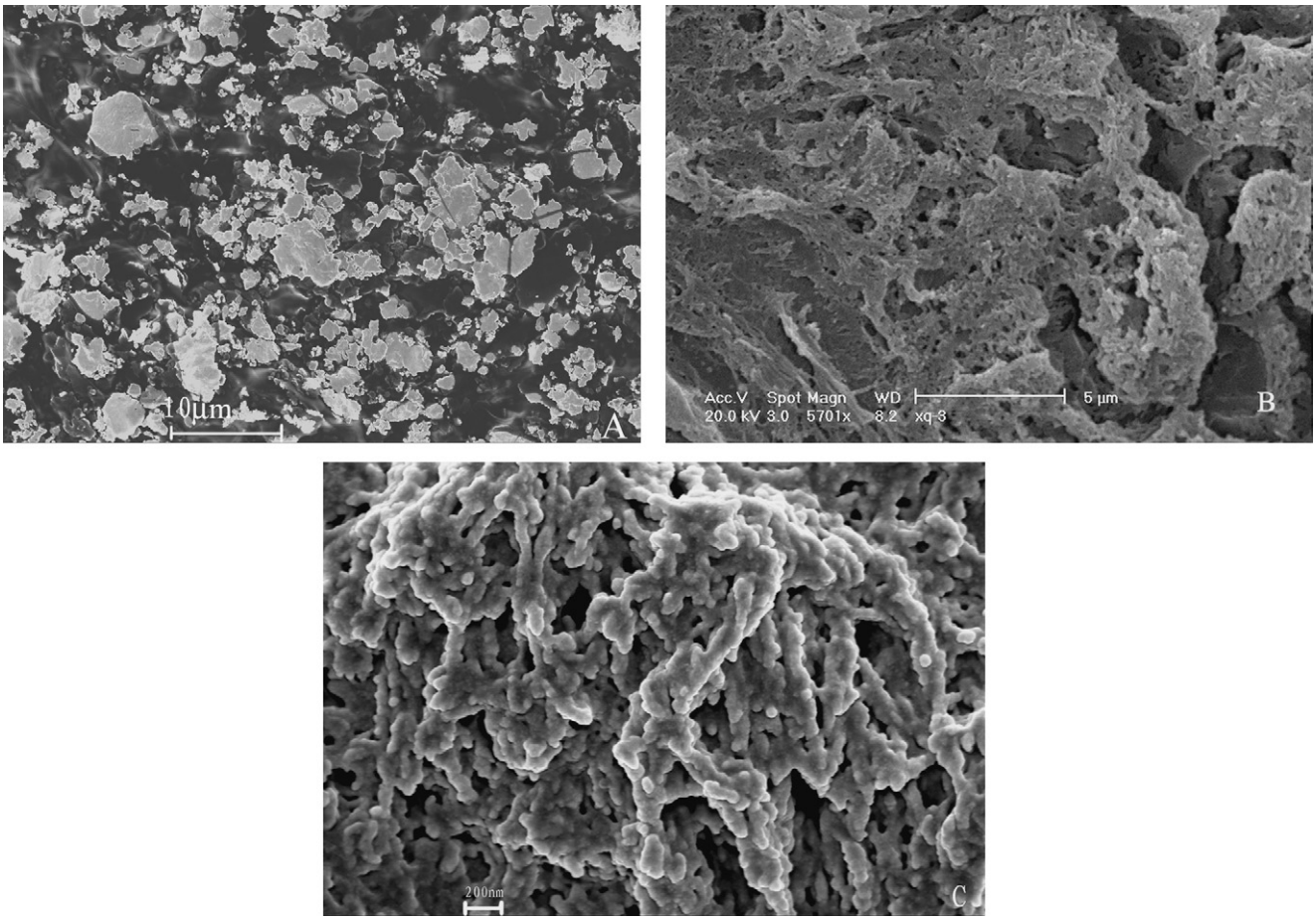

Fig. 1. SEM of PANI deposited from aqueous solution (A) and LCC templated mesoporous PANI (B and C).

film, and indicated a mesoscopic order with a d spacing of 5.8 nm.

The surface density of the electroactive units of PANI film were estimated from cyclic voltammetry experiments. Fig. 3 shows the voltammograms of two different kinds of PANI films in 1 M $HClO_4$ at the scan rate of 50 mV s^{-1}. Both samples showed the typical reduction and oxidation responses of PANI. It was attributed to the conversion of leucoemeraldine/emeraldine [23]. However, the mesoporous PANI film (Fig. 3b) has a considerably higher redox current and capacity, which indicates that more effective surface areas of the mesoporous PANI are accessible to the electrolytes. The surface density of the electroactive units of PANI (Γ) can be deduced from the

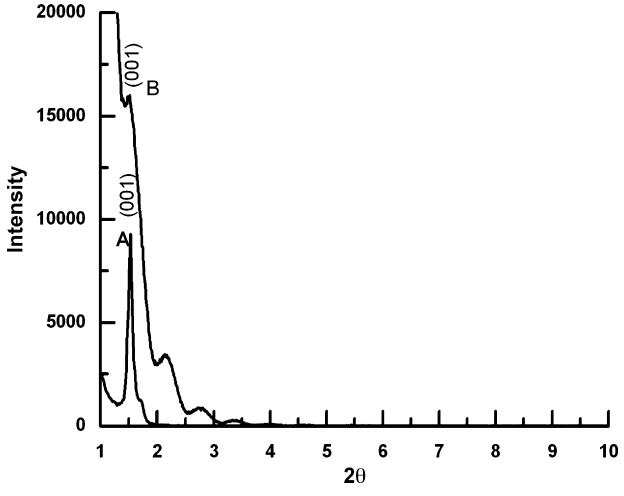

Fig. 2. Low-angle XRD patterns for LCC template aniline electrolyte (A) and mesoporous PANI films (B).

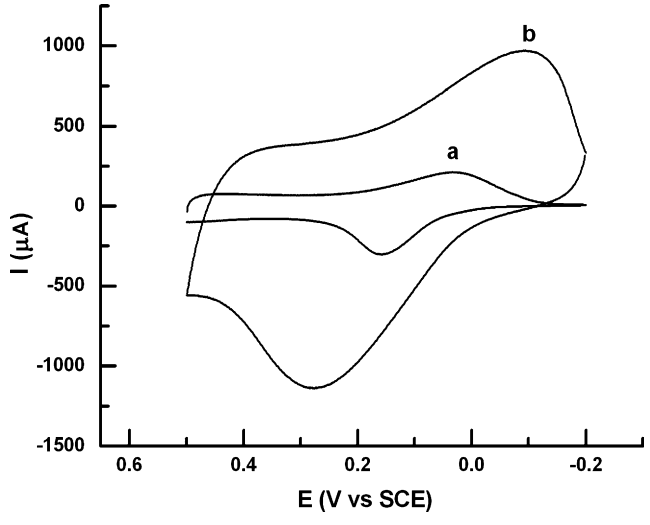

Fig. 3. Cyclic voltammetry of PANI deposited from aqueous solution (a) and LCC template mesoporous PANI (b) in 1 M $HClO_4$ at the scan rate of 50 mV s^{-1}.

following equation [24]:

$$\Gamma = \frac{4RTi_p}{n^2F^2\nu A}$$

where ν, A and i_p represent the scan rate, the electrode area, and the peak current, respectively. The surface density of the mesoporous PANI was estimated to be 3.0×10^{-7} mol cm^{-2}. However, the surface density of PANI deposited in the absence of Brij 56 was found to be about 6.5×10^{-8} mol cm^{-2}. It has been reported that the metal films obtained using the lyotropic liquid crystalline phases as the template combine well-defined porous nanostructures, high specific surface areas, electrical connectivity, and fast electrolyte diffusion [20,21]. These properties, together with a uniform pore size distribution, mechanical stability, and ease of processing, suggested that nanostructured mesoporous PANI films could be of considerable interest for biomolecule immobilization. The mechanism for the biomolecule immobilization could be explained as a combination of electrostatic and hydrophobic interactions [25,26]. At pH 7.0, the PANI films are expected to have negative surface charges (zero charge at pH 5.5), which favors electrostatic interactions with the cluster of positively charged HRP (p*I* 8.9). The immobilized HRP was reasonably stable; after an initial loss during the first 20 potential cycles of the electrode in pH 7.0 PBS solution, the signal remained identical for the next experiment.

In order to investigate the bioactivity of the adsorbed HRP, the fabricated electrode was employed as a biosensor for the electrocatalysis of hydrogen peroxide. Fig. 4 shows the bioelectrocatalytic behavior of the HRP–nonporous PANI (Fig. 4a) and HRP–mesoporous PANI film (Fig. 4c) modified electrodes in 0.1 M PBS 7.0 solution. In the presence of 2.0 mmol L^{-1} H_2O_2, the cathodic response of the HRP–mesoporous PANI film modified electrode (Fig. 4d) enhanced more remarkably than that of the HRP–nonporous PANI film modified electrode (Fig. 4b). Due to the large specific surface area of the mesoporous PANI film, more HRP molecules were immobilized, which contributed to the remarkable current of H_2O_2 on the HRP–mesoporous PANI film modified electrode. It is well-known that HRP often exhibits sluggish electron transfer at conventional electrodes because of its unfavorable orientation on the electrode surface or the adsorption of impurities to make it denature. In the previous reports, HRP-catalyzed reduction of peroxides usually occurred in the presence of redox species that were served as either electron donors or hydrogen donors [27]. Here, no additional electron transfer mediator was needed because of the electroactive character of PANI itself. It could be envisaged as an additional advantage over conventional polymer matrices for enzyme immobilization. The electron-donating ability of PANI may play an important role in enzyme catalysis. Pekmez explained the reduction of PANI film when the electrode was polarized at negative potentials, and the polymer was thus as a mixture of reduced and oxidized species. The PANI in its reduced form could be used as the electron transfer mediator. The substrate (H_2O_2) reduction charge is propagated along the polymer chain to the electrode surface by fast electron transfer reactions involving PANI$^{0/+}$ redox species [28].

The thickness of the mesoporous PANI film varies with the cycle numbers of CV scans. With the increase of the scan numbers, the thickness of mesoporous PNAI film increased too. It would increase the amounts of the immobilized HRP, which would enhance the response current of the biosensor to H_2O_2. However, the response time of the biosensor increased drastically with the increase of the thickness of the film. It may be due to the increase of the electrons transfer distance from the electrode surface to the redox center of the immobilized HRP. In our experiments we chose 100 cycles to obtain the optimized thickness of PANI film.

To ascertain the effect of pH on the response of the biosensor, the amperometric response of the HRP/PANI modified electrode to 10 μM H_2O_2 was recorded through successively adding H_2O_2 to a continuous stirring PBS solution with different pH, as shown in Fig. 5. It could be observed that the biosensor obtained its highest response at pH 5.0. With the increase of pH, the response currents decreased. At pH 9.0, almost no response current of the

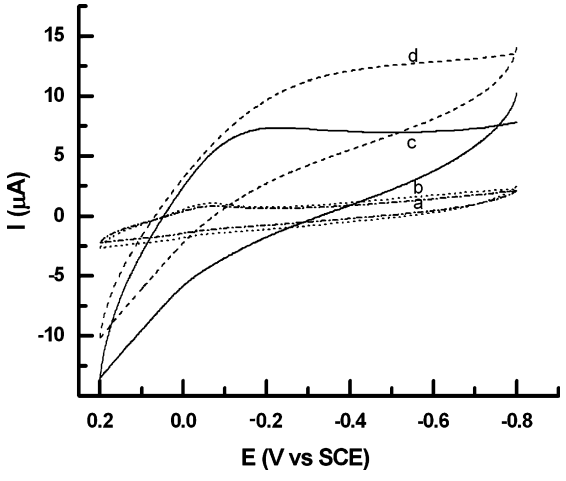

Fig. 4. Cyclic voltammograms of HRP–nonporous PANI film (a and b) and HRP–mesoporous PANI film (c and d) modified electrode in the absence (a and c) and presence of 2.0 mmol L^{-1} H_2O_2 in 0.1 M PBS 7.0 solution. Scan rate, 100 mV s^{-1}.

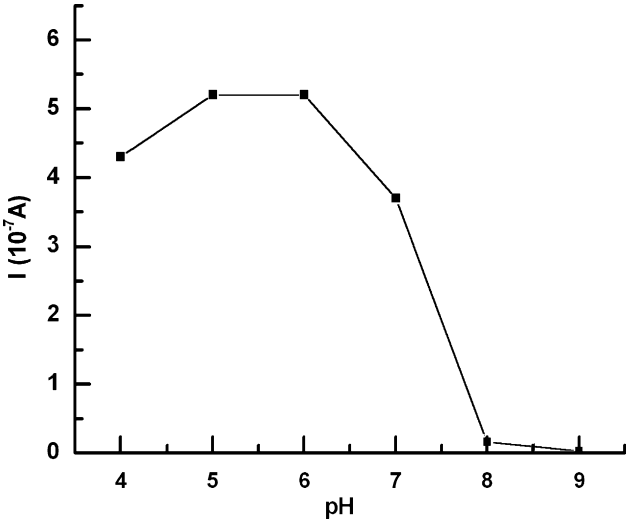

Fig. 5. Influence of pH values of PBS on the response currents of 10 μM H_2O_2 at the HRP–mesoporous PANI film modified electrode.

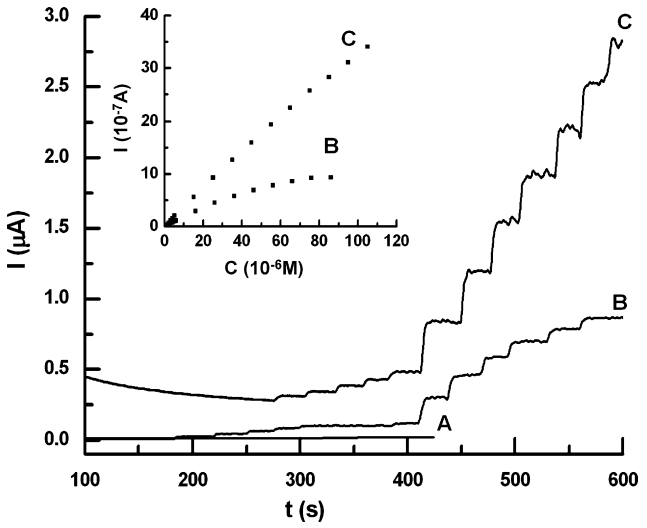

Fig. 6. Typical steady-state response of the biosensor on successive injection of different concentration of H_2O_2 into 0.1 M stirring PBS 7.0 solution. Applied potential, −100 mV (A, PANI; B, HRP–nonporous PANI; C, HRP–mesoporous PANI).

biosensor was observed. The better sensitivity of the biosensor at pH 5.0 may be due to the better conductivity of PANI in the acidic solutions. With the decrease of pH, the electronic conductivity of PANI increased [29]. However, the acidic solution would also denature HRP. Therefore, the response current of the biosensor decreased at pH 4.0 solution. For all practical purposes, the application of the biosensor in a neutral solution is an important aspect to pay attention to, so we selected pH 7.0 PBS solution for the detection of H_2O_2 in our experiments.

Fig. 5 shows the typical steady-state responses of three kinds of biosensors on successive injection of different concentration of H_2O_2. In the absence of HRP, a small catalytic current was observed even when 10^{-3} M H_2O_2 was added into the solution (Fig. 6A). However, after immobilizing HRP on the PANI films, the reduction current increased sharply, as shown in Fig. 6B and C. The reduction current at the HRP–mesoporous PANI film (Fig. 6C) enhanced more than 100% over HRP–nonporous PANI modified electrode (Fig. 6B). It indicated that the porosity of the PANI film ensured a large specific surface area and a high loading factor of enzyme per volume unit, which resulted in more HRP molecules to be immobilized on the mesoporous PANI film. The modified electrode achieved 95% of the maximum steady-state current in less than 10 s. The results demonstrated clearly that the electrocatalytic response was fast. The fast response might be due to the open structure of the porous film [30]. When the biomolecules were doped onto the surface of the polymer, the substrates or biorecognition molecules should not have to diffuse into and out of a polymer matrix. However, it occurs traditionally in polymer entrapment systems. Also, the speed at which the reactants reached the active centers in mesoporous organic–inorganic hybrids was reported to be higher in ordered mesostructures than in the amorphous ones [31]. The inset of Fig. 6 compares the calibration data for HRP–mesoporous PANI film modified electrode and an HRP–ordinary PANI film modified electrode over the range 1.0 to 100 μM. The limitation current of the HRP–nonporous PANI film modified electrode was not proportional to the concentration of hydrogen peroxide beyond 100 μM. If the added hydrogen peroxide concentration was over 100 μM, it would reach a plateau as the binding sites of HRP become saturated. In comparison, the mesoporous electrode response was linear up to ~2.0 mM. An increased surface area of the mesoporous PANI film electrode may provide more sites for HRP immobilization, and the binding sites for H_2O_2 increase. The detection limit of the sensor was 0.63 μM estimated at a signal-to-noise ratio of 3.

The reproducibility of the sensor was evaluated at a H_2O_2 concentration of 0.1 mM, and the relative standard deviation was 1.36% ($n = 10$). For the inter-electrode repeatability of ten electrodes from the same batch, the relative standard deviation was 2.34% at a H_2O_2 concentration of 0.1 mM. The stability of the sensor was investigated. The response remained stable after continuously detecting H_2O_2 for about 2 h. When the electrode was put in a drying state at 4 °C and measured everyday, the current response only decreased by 6% of the original value after 30 days.

4. Conclusion

In this work, an ordered nanostructured mesoporous PANI film with the high surface area, small nanofiber diameter, and ordered porous nature was synthesized by using LCC as the template. The conductivity character of PANI made it be used as an electron-relaying polymer to wire the electron transfer between enzymes with the electrode. The obtained film offers a good environment for enzyme loading as well as substrate diffusion, resulting in high sensitivity, wide linearity and long-term stability of the biosensor. It opens a new doorway for the application of PANI as bioanalytical devices.

Acknowledgements

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