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Short communication

Graphitized macroporous carbon microarray with hierarchical mesopores as host for the fabrication of electrochemical biosensor

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ABSTRACT

A novel graphitized ordered macroporous carbon (GMC, pore size ~380 nm) with hierarchical mesopores (2–30 nm) and high graphitization degree was prepared by nickel-catalyzed graphitization of polystyrene arrays. The obtained GMC possessed high specific surface area, large pore volume, and good electrical conductivity, which was explored for the enzyme entrapment and biosensor fabrication by a facile method. With advantages of novel nanostructure and good electrical conductivity, direct electrochemistry of hemoglobin (a model protein) was observed on the GMC-based biocomposite with a formal potential of −0.36 V (vs. Ag/AgCl) and an apparent heterogeneous electron transfer rate constant (k_s) of 1.2 s^{-1} in pH 7.0 buffer. Comparative studies revealed that GMC offered significant advantages over carbon nanotubes (CNTs) in facilitating direct electron transfer of entrapped Hb. The fabricated biosensor exhibited good sensitivity (101.6 mA cm^{-2} M^{-1}) and reproducibility, wide linear range (1–267 μM), low detection limit (0.1 μM), and good long-term stability for H_2O_2 detection. GMC proved to be a promising matrix for enzyme entrapment and biosensor fabrication, and may find wide potential applications in biomedical detection and environmental analyses.

1. Introduction

The fundamental aspects of an electrochemical biosensor involve the enzyme immobilization onto an electrode surface and the formation of efficient electrical communication between the enzyme and the electrode while retaining the enzymatic stability and bioactivity (Willner et al., 2007; Zang et al., 2007). To achieve this goal, one promising way is to employ porous nanomaterials (Lu et al., 2008a; Zeng et al., 2007; Zhou et al., 2008; Wang et al., 2009; Ispas et al., 2009), especially mesoporous carbon (2–50 nm, Lee et al., 2005; Feng et al., 2007; Wu et al., 2007), as favorable host for the entrapment of enzymes. The direct electron transfer has been achieved on these porous nanomaterials, which offer opportunities for the construction of superior third-generation biosensor, as it obviates the need for mediators and allows efficient transduction of the biorecognition event (Wang, 2005).

Graphitized ordered macroporous carbons (>50 nm, Lei et al., 2007; Stein et al., 2009), which are typically prepared by a template-assisted method, are of great interest in biosensing area as they can combine properties of high specific surface area, large pore volume, and good electrical conductivity and stability. The three-dimensional ordered macropores earned by graphitized macroporous carbons can be used as favorable immobilization matrix to accommodate enzymes, meanwhile their open framework can provide convenience for the mass transfer of enzyme-catalyzed substrate and product. Furthermore, the good electrical conductivity of graphitized macroporous carbons enables them to act as nano-scaled transducer for the biorecognition events. These unique properties of graphitized macroporous carbons offer excellent prospect for their application in electrochemical biosensors. However, up to date, the biosensing applications of graphitized macroporous carbons are rarely reported (Dimakis et al., 2002; Zhang, 2008).

One major problem limits the application of graphitized macroporous carbons is the poor dispersion in aqueous solution. In previous pioneering studies, the macroporous carbon was squeezed into a tube (e.g. polytetrafluoroethylene) to form a carbon rod electrode, and the enzyme molecules were immobilized on the carbon electrode by physically absorbing in the enzyme solutions (Dimakis et al., 2002; Zhang, 2008). The primary disadvantages of the above method lie in their poor and non-controllable enzyme load, and the complicated and time-consuming enzyme immobilization process (typically 16 h or 20 h). In the present study, a novel graphitized ordered macroporous carbon (denoted as GMC) with hierarchical mesopores and high graphitization degree was prepared by nickel-catalyzed graphitization of polystyrene arrays, which was explored for the first time as host for enzyme

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entrapment and biosensor fabrication by a facile method. The problem of poor dispersion of GMC in aqueous solution was solved by surface modification of GMC with Nafion (a perfluorosulfonate linear polymer with good conductivity and biocompatibility, Nadzhafova et al., 2004). The high electronic conductivity and favorable nanostructure (simultaneously possesses abundant of macropores and mesopores) of the GMC-based nanocomposite, coupled with its controllable enzyme load, improved biocompatibility and ease of processing, make it extremely attractive as biosensing materials. Hemoglobin, an important respiratory protein that has peroxidase-like activity, was chosen as a model protein to study the biosensing performance of GMC. The advantages of GMC were illustrated from comparison to CNTs, and results revealed that GMC has significant advantages over CNTs in facilitating the electron transfer of entrapped Hb and enhancing the performance of the fabricated biosensor.

2. Experimental

2.1. Materials

Hemoglobin (from bovine blood) and Nafion solution (5 wt.% in lower aliphatic alcohols) were purchased from Sigma. CNTs (purity > 99.9%, graphitized at 2800 °C) were from Chengdu Institute of Organic Chemistry (China). GMC was prepared according to our previously reported method (Lei et al., 2007). All other reagents were of analytical-grade. All solutions were prepared with Milli-Q water (>18.0 MΩ cm).

2.2. Preparation of enzyme electrodes

The enzyme electrode was prepared by a simple casting method. Firstly, 1.2 mg ml^{-1} GMC suspension was obtained by adding 1.2 mg of as-prepared GMC into 1 ml Nafion solution (5 wt.%) with the aid of ultrasonication. Then, a suspension (denoted as Suspension I), which finally contained 2.5 mg ml^{-1} Hb, 0.4 mg ml^{-1} GMC and 1.67% Nafion, was prepared by mixing appropriate volume of Hb solution (10 mg ml^{-1}, dissolved in 25 mM pH 7.0 phosphate buffer) and GMC suspension thoroughly. Finally, 4 μl of Suspension I was cast onto the surface of a freshly polished glassy carbon electrode (GC, 3 mm diameter) to prepare the Hb–Nafion–GMC/GC electrode. A beaker was covered over the electrode so that water can evaporate slowly in air and a uniform film electrode can be obtained. The dried Hb–Nafion–GMC/GC electrode was stored at 4 °C in pH 7.0 phosphate buffer when not in use.

Other enzyme electrodes were prepared with similar procedures as described above. The suspension containing 2.5 mg ml^{-1} Hb, 1.67% Nafion and 0.4 mg ml^{-1} CNTs was used to prepare the Hb–Nafion–CNTs/GC electrode, and the solution containing 2.5 mg ml^{-1} Hb, 1.67% Nafion was used to prepare the Hb–Nafion/GC electrode. Before electrochemical measurements, all the as-prepared enzyme electrodes were immersed in pH 7.0 phosphate buffer for 30 min to remove physically absorbed components.

2.3. Apparatus and measurements

TEM images were taken with a transmission electron microscope JEM-2000EX (JEOL, Japan) with an accelerating voltage of 120 kV. SEM images were taken with a scanning electron microscope JSM-6360 (JEOL, Japan). Nitrogen adsorption–desorption isotherms were measured at 77 K using a Quantachrome NOVA 4000 analyser (Quantachrome, USA). The specific surface area of the sample was calculated by the Brunauer–Emmett–Teller (BET) method, and the pore size distribution was calculated from the desorption branch using the Barrett–Joyner–Halenda (BJH) method. Powder X-ray diffraction (XRD) was carried out using a X'Pert PRO X-ray diffractometer (PANalytical, Holland) with Cu Kα radiation (λ = 1.54178 Å). Electrochemical measurements were performed at room temperature using a CHI 440 workstation (CH Instruments, Inc., Austin, USA). The measurements were based on a three-electrode system with the prepared enzyme electrode as the working electrode, a platinum wire as the auxiliary electrode, and an Ag/AgCl (3 M KCl) electrode as the reference electrode. Unless otherwise noted, 50 mM pH 7.0 phosphate buffer was deoxygenated and used as the electrolyte in all experiments.

3. Results and discussion

3.1. Characterization of the prepared GMC

GMC was formed by catalytic graphitization of polystyrene arrays, in which polystyrene arrays were used as both template and carbon source for the generation of macroporous composite (Lei et al., 2007). Fig. 1a shows the XRD patterns of GMC. As shown, the intensive peaks at 26° and 44.5° could be indexed to the (0 0 2) and (1 0 0) diffractions of the graphite, revealing high crystallinity and graphitization degree of GMC. The high graphitization degree of GMC revealed the good electrical conductivity, which was essential for the electrical transducing of biorecognition events. Fig. 1b and c shows the TEM and SEM images of the prepared GMC after removal of SiO_2 by HF acid solution. It can be seen that the GMC had a three-dimensional ordered spherical macroporous structure with an average pore diameter of ~380 nm. These abundant macropores were close-packed, forming an ordered microarray. The wall thickness was estimated to be 10–20 nm.

The microstructure of GMC was further characterized by nitrogen adsorption-desorption isotherms. As shown in Fig. S1, the evident hysteresis loop corresponded to the capillary condensation in the mesopores, suggesting the existence of textural mesopores on the macroporous wall of GMC. The BET specific surface area of GMC was calculated to be 150 m^2 g^{-1}, and the total pore volume for pores with diameter less than 129 nm was 0.367 cm^3 g^{-1} (The total pore volume of GMC was actually far larger than this value considering the average pore size of 380 nm). The BJH pore size distribution is also shown in Fig. S1, revealing the mesopore size of GMC mainly focused in the range of 2–30 nm. The removal of SiO_2 from the SiO_2/graphitized carbon by HF solution left behind many mesopores, which contributed to the mesoporous characterization of GMC. These large amounts of mesopores together with the macropore array could offer favorable microenvironment for the entrapment of enzyme molecules, meanwhile could provide convenience for the mass transfer of enzyme-catalyzed substrate and product.

3.2. Direct electron transfer of the enzyme electrode

Fig. 2 shows typical cyclic voltammograms of different enzyme electrodes in 50 mM pH 7.0 phosphate buffer. A pair of stable and well-defined redox peaks of Hb for the Hb(Fe^{III})/Hb(Fe^{II}) redox couple transformation, which could be ascribed to the direct electron transfer between the Hb and the underlying electrode, were observed at the Hb–Nafion–GMC/GC electrode (Fig. 2a). The formal potential $E^{\circ\prime}$ of Hb was −0.36 V (vs. Ag/AgCl), characteristic of Hb($Fe^{III/II}$) redox couples in various films (Lu et al., 2007, 2008b). The potential difference ΔE_p between the anodic and cathodic peak potentials, which is directly related to the electron transfer rate, was about 51 mV (when the scan rate was 0.1 V s^{-1}). Such a small ΔE_p value revealed a fast and quasi-reversible electron transfer process. Fig. S2 (the supplementary information) demonstrates the cyclic voltammograms of Hb–Nafion–GMC/GC electrode with scan rates from 0.1 to 1.0 V s^{-1}. As shown, the cathodic and anodic

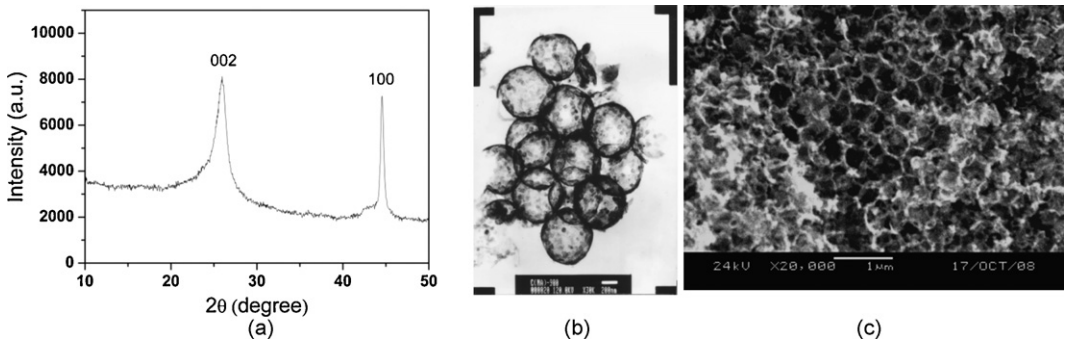

Fig. 1. (a) XRD patterns, (b) TEM and (c) SEM images of the prepared GMC.

peak currents increased linearly with scan rate from 0.1 to 1.0 V s^{-1}. This revealed that the electron transfer between Hb and GC electrode was a surface-controlled electrochemical process. According to the Laviron method for a surface-controlled electrochemical system (Laviron, 1979), the apparent heterogeneous electron transfer rate constant (k_s) of Hb immobilized on Hb–Nafion–GMC/GC was estimated to be about 1.20 s^{-1}, which was comparable to that of Hb immobilized on Hb–Nafion–CNTs/GC (1.18 s^{-1}), suggesting a fast electron transfer process. There was a pair of negligible redox peaks at about −0.15 V at the Hb–Nafion–GMC/GC electrode, which was almost consistent with that observed at Nafion/GC (not shown) and possibly caused by the electrode matrix Nafion. Compared with Hb–Nafion–GMC/GC, the redox peaks observed at the Hb–Nafion/GC (Fig. 2c) were negligible. It was prominent from the comparison that direct electron transfer between Hb molecules and GC electrode was greatly enhanced by GMC in the composite film. The possible reasons could be elucidated as follows. Firstly, due to its high surface area and good electrical conductivity, GMC could dramatically enhance the active surface area of the GC electrode available for protein binding; meanwhile, it could act as nano-scaled electrical transducer to facilitate the electron transfer between the redox proteins and the underlying electrode. Moreover, the porous framework might provide a protective microenvironment for entrapped proteins to prevent denaturation or inactivation.

In comparison with Hb–Nafion–GMC/GC, the redox peaks at Hb–Nafion–CNTs/GC were much smaller. According to Faraday's law, $Q = nFA\Gamma^*$ (where F is Faraday constant, Q can be obtained by integrating the reduction peak of Hb, n and A stand for the electron transfer number and the geometrical surface area of the electrode, respectively), the surface concentration of electroactive Hb (Γ^*) at Hb–Nafion–GMC/GC was estimated to be 5.11×10^{-10} mol cm^{-2} (assuming an one-electron transfer reaction). This value was about three times larger than that obtained at Hb–Nafion–CNTs/GC (1.73×10^{-10} mol cm^{-2}), and much larger than the reported value of Hb on porous Co_3O_4 nanosheet (Lu et al., 2007) or carbon nanofiber-based electrode (Lu et al., 2008b). It should be noted that the protein load on different enzyme electrodes was equal. The percentage of electroactive Hb on Hb–Nafion–GMC/GC was as high as 23.3% (the total amount of Hb on electrode surface can be roughly estimated by the amount of protein loaded (i.e. the Hb amount in 4 μl Suspension I containing 2.5 mg ml^{-1} Hb), as the leakage of loaded Hb from the freshly prepared enzyme electrode to the buffer solution was neglectable (far less than 10% based on the UV–vis spectroscopic analysis (not shown)), this value was much larger than that on Hb–Nafion–CNTs/GC (7.9%), revealing that the entrapped Hb on GMC can easily perform electron transfer with the underlying electrode. By comparison, it can be concluded that GMC offered significant advantages over CNTs and other immobilization matrix in facilitating direct electron transfer of Hb. It should be emphasized that the enhancement in direct electron transfer should not be limited to the electron transfer rate constant. The enhancement in electroactive concentration and electroactive percentage was also one important aspect of direct electron transfer of Hb, which contributes to the good performance of the biosensor. Although the apparent heterogenous electron transfer rate constants of Hb on GMC and CNTs had no significant difference, the electroactive concentration and electroactive percentage of Hb on GMC were much larger than that on CNTs. These might result from the larger effective surface area of GMC than that of CNTs, and the better microenvironment of Hb sequestered in the macropores and mesopores of GMC compared to that of Hb absorbed on the exterior surface of CNTs.

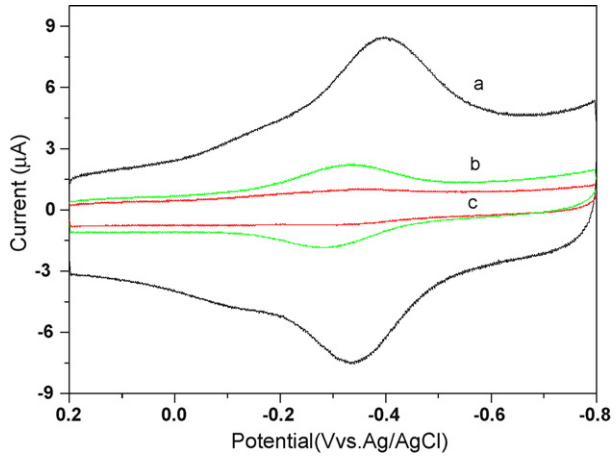

Fig. 2. Cyclic voltammograms of (a) Hb–Nafion–GMC/GC, (b) Hb–Nafion–CNTs/GC and (c) Hb–Nafion/GC in pH 7.0 phosphate buffer with scan rate of 0.2 V s^{-1}.

3.3. Amperometric response of the enzyme electrode for hydrogen peroxide

As is well known, H_2O_2 is an important biological intermediate, and the determination of H_2O_2 is of great importance in many fields, such as in clinical, food, pharmaceutical and environmental analyses. In our experiments, we used amperometry for the detection of H_2O_2, which can minimize the disturbance of the large capacitive current of GMC in cyclic voltammetry. The cathodic peak potential of H_2O_2 on the enzyme electrode (−0.388 V) by cyclic voltammetry was selected as the applied potential of amperometry for the high sensitivity. Fig. 3 demonstrates the typical current–time curves of the biosensor upon successive addition of H_2O_2 to the stirring buffer at a constant stir rate. The response time (achieving 95% of the steady-state current) was less than 3 s. Such a rapid response can be attributed to the fast diffusion of the substrate in the porous enzyme electrode. The inset in Fig. 3 shows the corresponding cal-

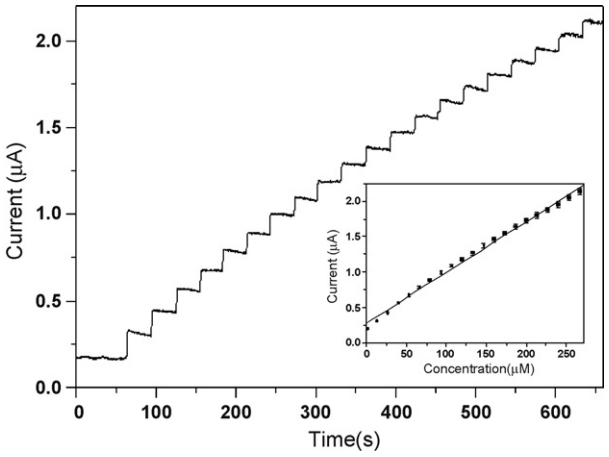

Fig. 3. Typical amperometric current–time response curves of Hb–Nafion–GMC/GC upon successive addition of H_2O_2 into pH 7.0 phosphate buffer. Inset: the corresponding calibration curves of steady-state currents vs. H_2O_2 concentrations.

ibration curve of the steady-state current vs. H_2O_2 concentration. As shown, the steady-state response current increased linearly with the increasing H_2O_2 concentration. The linear range was from 1 to 267 μM with a sensitivity of 101.6 mA cm^{-2} M^{-1} (n = 21, R = 0.998). The detection limit was as low as 0.1 μM (S/N = 3). Further increasing the H_2O_2 concentration resulted in a rise in the catalytic current up to a limiting value; such saturation behavior was characteristics of enzyme-based catalysis. The apparent Michaelis–Menten constant (K_M^{app}) was estimated to be 180 μM according to the electrochemical version of Lineweaver–Burk equation (Kamin and Wilson, 1980). This value was comparable to those ever reported values (Lu et al., 2007), indicating that Hb entrapped in the composite film possessed high peroxidase activity.

3.4. Reproducibility, stability and selectivity of the enzyme electrode

The reproducibility, stability and selectivity of the enzyme electrode were evaluated by cyclic voltammetry in the presence of H_2O_2. The RSD of intraelectrode repeatability never exceeded 2.6% (n = 5), while the RSD of interelectrode reproducibility was around 5%. The prepared enzyme electrode was stored in pH 7.0 buffer at 4 °C when it was not in use. The enzyme electrode could retain more than 90% of its initial response after a 21-day storage, demonstrating good long-term stability. The abundant macropores and mesopores earned by GMC coupled with the good biocompatibility of Nafion, provided a favorable microenvironment for entrapped enzyme molecules to retain their bioactivity. The major coexisting species in biomedical sample such as ascorbic acid, uric acid (with the same concentration as H_2O_2) and glucose (5 mM) did not interfere with the detection of H_2O_2, which might be attributed to the intrinsic selectivity of Hb for its substances and the permselectivity of Nafion.

4. Conclusions

Graphitized ordered macroporous carbon with hierarchical mesopores was successfully used for the entrapment of model protein Hb to fabricate a mediator-free biosensor by a facile way. Benefiting from the excellent conductivity and favorable nanostructure, the entrapped Hb displayed facilitated direct electron transfer with the underlying electrode and high peroxidase-like activity toward H_2O_2. The fabricated biosensor exhibited good sensitivity and reproducibility, wide linear range, low detection limit, and good long-term stability. The graphitized ordered macroporous carbon with hierarchical mesopores could be used effectively for the entrapment of redox-active enzymes, and may find wide potential applications in biomedical detection and environmental analyses.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2009.06.018.

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