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Enzyme immobilization on amino-functionalized mesostructured cellular foam surfaces, characterization and catalytic properties

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Abstract

Large mesopores cellular foam (LMCFs) materials with diameters ranging from 17 to 34 nm were synthesized using microemulsion templating. The amine functional groups were attached to channels of LMCFs materials via post-synthesis grafting. The structural and chemical properties of these prepared materials were characterized by TEM, XRD, FTIR and nitrogen adsorption. The glucose oxidase (GOx) was immobilized by covalently couple enzyme molecules to the interior surface of amino-functionalized mesostructured cellular foams (AF-MCFs) materials, in which leaching of the enzyme is prevented. The immobilized enzyme exhibited the high catalytic activity and thermal stability for oxidation of glucose. It was found that GOx immobilized on AF-MCFs materials is re-useable.

Keywords: Enzyme immobilisation; Functionalized mesoporous materials; Glucose oxidase; Enzyme stability; Catalytic activity

1. Introduction

The specific immobilization of enzymes onto solid supports is of considerable interest for their applications as enzyme reactors, biological fuel cells and biosensors [1,2]. Functionalized mesoporous materials with high surface area, controllable pore size, narrow pore size distribution, thermal and mechanical stability and featuring high functionality, provide high affinity with the enzyme molecules leading to high enzyme loading [3–5]. Several periodic mesoporous materials with uniform pore diameters of 2–15 nm such as MCM-41, MCM-48, M41S and BAS have been used for the enzyme immobilizations. The enzyme stability can be enhanced by immobilizing enzyme on mesoporous materials [3,6,7].

In biotechnological applications, the use of high-molecular-weight materials requires mesoporous materials with well-defined large mesopores (15–50 nm) or macroporous pores (>50 nm) for the diffusion of reagents and products through pore channel in order to avoid undesired pore blocking phenomena [8–11]. A considerable effort has been devoted to develop large mesopores and macroporous materials as supports for the immobilization of enzyme. The synthesis of mesostructured cellular silica foams has been studied by several researchers [12–16]. The major advantage of the combination of surfactant templating techniques and emulsion or microemulsion templating is that it provides a simple and effective way to fabricate mesoporous materials with well-defined pore structures from mesoporous to large and ultralarge. Therefore, functionalized large mesopores materials with a high density of amino groups and well-defined mesochannels have resulted in a revolution of the use of biomolecule for applications in separation, catalysis and sensors, which typically depends on the successful immobilization of biomolecule onto suitable host [17,18]. Despite its importance for the biotechnology applications, the preparation and applications for the immobilization of enzyme of functionalized mesostructured cellular foams with

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large mesopores by oil/water microemulsion templating has not been reported.

In this paper, we report the synthesis of amino-functionalized mesostructured cellular foams (AF-MCFs) with large mesopores by the post synthetic grafting of tetraethoxysilane (TEOS) and 3-aminopropyl-triethoxysilane (ATES) using the microemulsion templating. AF-MCF materials prepared were characterized by transmission electron microscopy (TEM), X-ray diffraction (XRD), Fourier transform infrared (FTIR) and nitrogen sorption. Glucose oxidase (GOx) immobilized on AF-MCF materials and the performances of immobilized enzyme were investigated and discussed.

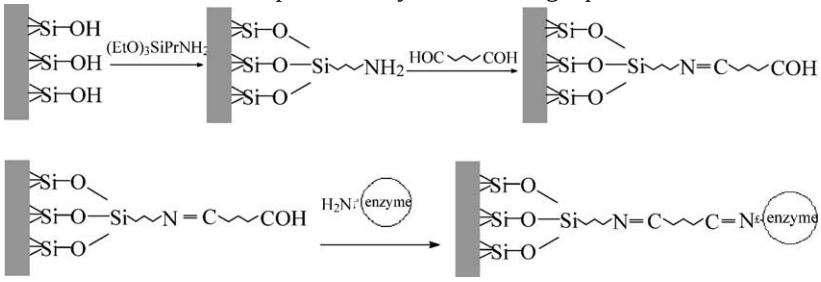
2. Experimental

Tetraethoxysilane, and EtOH were obtained from Aldrich. 3-Aminopropyl-triethoxysilane was obtained from ACROS. All chemicals were used as received. Water used in all synthetic procedures was deionized to 18 MΩ cm. Large mesopores cellular foam (LMCFs) materials were synthesized using microemulsion templating in acidic solutions. LMCF materials were prepared as follows: P123 (poly (ethylene oxide)-block-poly (propylene oxide)-block-poly (ethylene oxide), EO_{20}-PO_{70}-PO_{20}, M_{av} = 5800) of 2 g (0.4 mmol) was dissolved in 75 ml 1.5N HCl solution at room temperature with stirring. 1,3,5-Trimethylbenzene (TMB, 8 g, 68 mmol) as the organic solvent (oil phase) was then added to the surfactant solution with stirring to form an oil-in-water microemulsion. The amount of TMB used was controlled by the weight ratios of TMB to P_{123} from 0 to 10, a typical ratio was 4:1. The TEOS (TEOS: 4.4 g, 21 mmol) were then added to the surfactant solution and this mixture was covered and stirred at 38–40 °C for 24 h. The synthesized materials were aged at 110 °C for 24 h in an autoclave. These as-synthesized materials were rinsed with deionized water and dried at air for 2 days. These synthesized productions were calcined at 500 °C for 5 h in air to remove surfactant and form LMCF materials. For post-synthesis functionalization of LMCF materials, 0.2 g of calcined LMCF sample was suspended in 20 ml of ethanol. Then, appropriate amount of 3-aminopropyl-triethoxysilane was added to this silica suspended solution, in which the molar ratio of ATES to TEOS was controlled from 0 to 2. This reaction mixture was refluxed at 85 °C for 8 h. The white solid was filtered off, washed with deionized water and ethanol and dried at 60 °C for 24 h.

The AF-MCFs materials were characterized by a JOEL 2000FX transmission electron microscope. Small-angle X-ray diffraction measurements were performed on an X-ray diffractometer (Rigaku Rotflex D/Max-C) using Cu Kα radiation (λ = 0.15064 nm). Nitrogen adsorption measurements were carried out with a Tristar 3000 Micromeritics at 196 °C. Each sample was degassed for 2 h at 200 °C under vacuum of about 10^{-3} Torr in the degas port of the adsorption apparatus before being analyzed. Surface areas were calculated by the Brunauer–Emmett–Teller (BET) method [19]. The pore size was calculated using Barrett–Joyner–Hatenda (BJH) model [20]. Infrared spectra were measured on KBr disks with Thermo Nicolet (US) Avatar 360 FTIR spectrometer.

2.1. Enzyme immobilization

The glucose oxidase was used to investigate immobilization on the amino-functionalized mesostructured cellular foams materials. First, AF-MCFs materials react with amyl aldehyde to change amino groups into aldehyde groups, which can covalently couple enzyme molecules to the interior surface of AF-MCFs materials through the amino groups of enzyme interacting with the aldehyde groups. The reaction can be represented by the following equation:



Then, the 8 mg of mesoporous solid was suspended in 4 ml of enzyme solution (enzyme concentration: 1 mg/ml) and was shaken overnight at room temperature. Quantification of amino groups loaded on AF-MCFs materials was performed by Kjeldhl method [21]. The amount of enzyme adsorbed was measured by analyzing the enzyme content of the supernatant liquid using Bradford method [22]. The amount of GOx adsorbed on the mesoporous solid was calculated by deducting the concentration of supernatant from precursor solution.

2.2. Activity test

GOx activity was measured by catalyzing glucose at 0.1 mol/l phosphate buffer (pH 5.6) changing to gluconic acid according to the following equation:

$$\text{glucose} \xrightarrow{\text{GOx}} \text{gluconic acid} + 2e^-$$

The activity of glucose oxidase immobilized on the amino-functionalized mesostructured cellular foams materials was determined by titrating gluconic acid produced with NaOH solution to an initial pH value. Based on the consumption of NaOH in the first 10 min, the amount of gluconic acid produced was obtained, from which the activity of the immobilized enzyme can be calculated. The specific catalytic activity was expressed as micromoles gluconic acid produced per microgram of GOx at per min. The thermal performances of immobilized enzyme and free enzyme were investigated and compared by heating the solution to different temperature and maintaining for 60 min. Enzyme re-use was examined by filtered samples and washed with buffer solution and dried at 4 °C after the first test.

3. Results and discussion

Fig. 1 shows a typical TEM image of amino-functionalized mesostructured cellular foam (AF-MCF) materials at large mesopores. These AF-MFC materials possess a disordered mesoporous structure consistent with the structural features of MCFs reported by previous studies [12–16]. Larger mesopores with diameters ranging from 17 to 34 nm were observed from Fig. 1 and the wall thickness of the AF-MCFs was estimated to be from 2.5 to 4 nm by using TEM. Low angle X-ray diffraction (XRD) was performed for the amino-functionalized MFC materials to analyze the structure of the mesoporous materials. Fig. 2 shows the XRD peak in the low

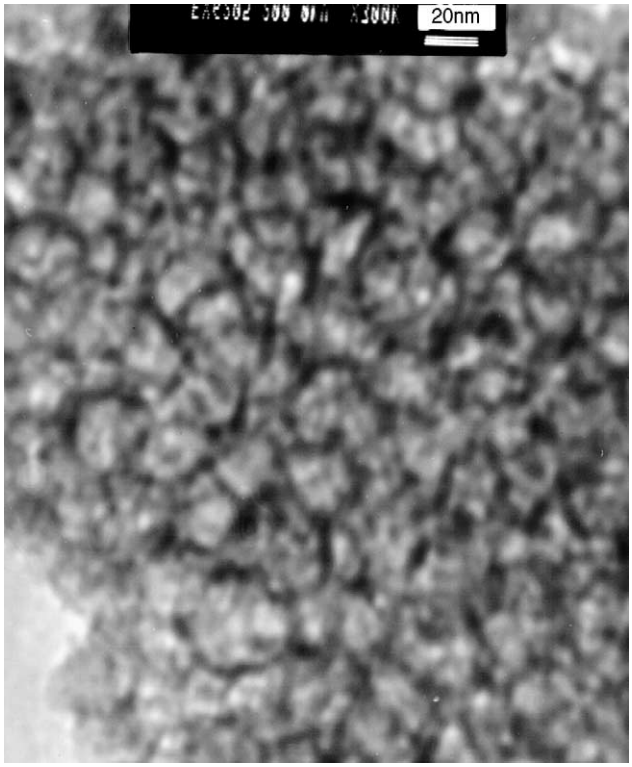


Fig. 1. Transmission electron microscope (TEM) image of the amino-functionalized mesostructured cellular foam (AF-MCF) materials prepared by microemulsion templating after surfactant removing.

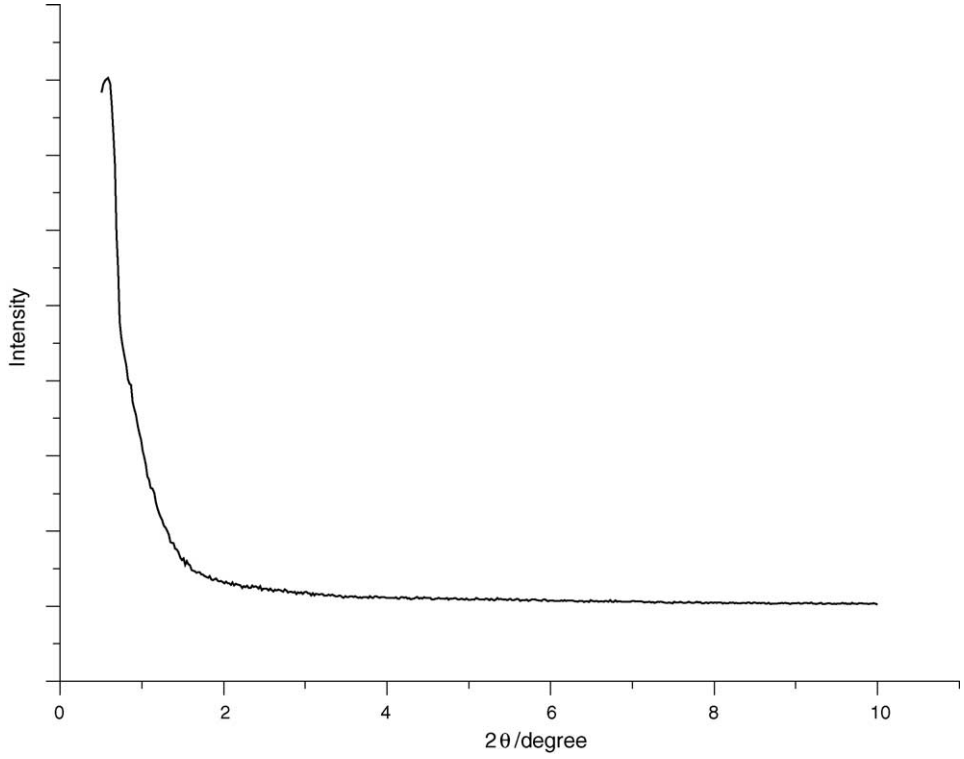


Fig. 2. X-ray diffraction patterns for the amino-functionalized mesostructured cellular foam (AF-MCF) materials prepared by microemulsion templating after surfactant removing.

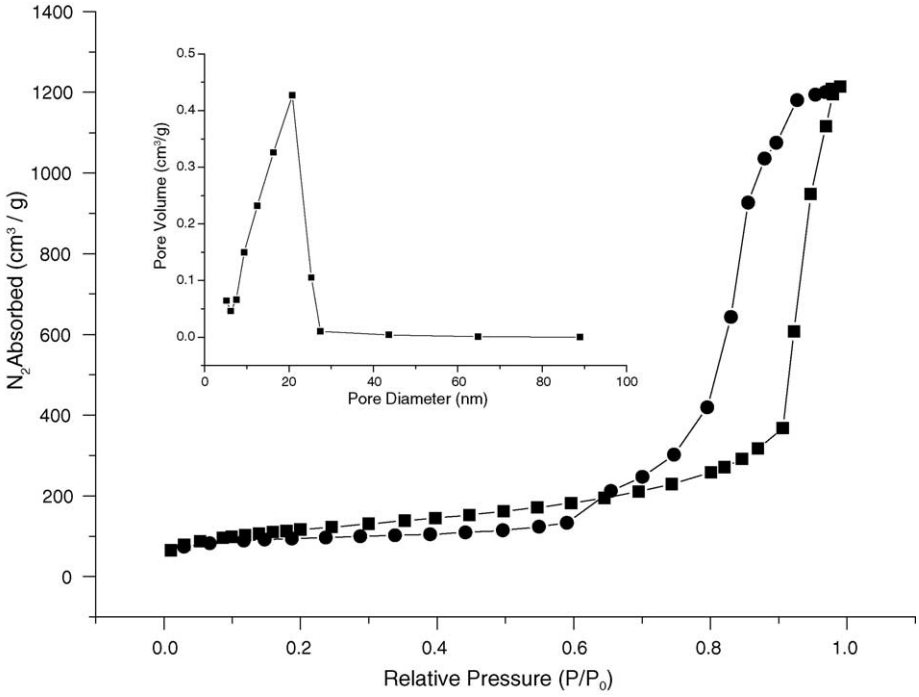


Fig. 3. Nitrogen adsorption isotherms and corresponding pore size analyses for the amino-functionalized mesostructured cellular foam (AF-MCF) materials prepared by microemulsion templating after surfactant removing.

angle region after calcinations, which indicates the presence of a mesoporous structure without any long range ordering [17,18]. The XRD pattern of large meso and macropore materials indicates only information on the intrananoparticle mesopores due to the large dimensions of the motive and the disordered characterization of these materials [23]. The pore size and pore structure of AF-MCF materials depend on the composition of precursor and preparation conditions. The ratio of mesitylene to surfactant (P_{123}) has a significant effect on the pore size and size distribution in the AF-MCFs. The average pore size increases with increasing ratio of mesitylene (TMB) to surfactant (P_{123}). The appropriate weight ratio of mesilylene to P_{123} in 4:1 was used to obtain the range of pore size from 17 to 34 nm in this study. Another factor that influences the pore size and pore structure is the aging time and aging temperature. The average pore size increases and the pore size distribution narrows with increasing aging time at 100–110 °C. The experimental results indicate that the acid preparation condition allows slow and moderate condensation of the TEOS, and the rates of condensation of TEOS can be controlled in microemulsion templating. A representative nitrogen adsorption–desorption isotherm of the amino-functionalized MFC materials is presented in Fig. 3. The isotherm is a type IV curve of mesoporous materials. A steep hysteretic loop is observed from this curve, which is typical for mesoporous materials that exhibit capillary condensation and evaporation [24,25]. The sharp rise in the nitrogen adsorption–desorption isotherms at high relative pressures (P/P_0 near 1) indicates the existence of large mesopores and macropores in these materials [16,26]. Fig. 3 (inset) shows the pore size distributions measured by the Barrett–Joyner–Hatenda model. The BET surface area is 520.7 m^2/g and the total pore volume is 1.38 cm^3/g. It is known that the large mesoporous and macroporous materials have lower surface area and higher pore volume than small mesoporous materials [27,28].

The presence of the amino-group onto the large mesopores framework after post-synthesis functionalization of LMCF materials has been confirmed by FTIR spectra. After post-synthesis, the C–N stretching vibration at 1180 cm^{-1} overlapping with Si–O–Si band at 1054 cm^{-1} and the symmetrical $-NH_3^+$ bending band at 1505 cm^{-1} can be observed from Fig. 4 and the weak symmetrical $-NH_3^+$ vibration at 1625 cm^{-1} overlapped with C=C band [11,29,30]. These amine function groups do not exhibit by MCFs materials prepared by TEOS alone as shown in Fig. 4a. This is evidence that the amino functional groups can bond to the interior mesoporous surfaces by this preparation method. The N–H absorption bands overlap with O–H bands at 3300–3500 cm^{-1} [5,11]. The strong Si–O–Si band at 1054 cm^{-1} and three weak bands at 940, 794 and 444 cm^{-1} belonged to the silica framework vibration bands consistent with a previous report [11,31]. There were a small observable line in C–H vibrations at 2880 cm^{-1}, but no peaks in the C–O–C vibrations at 1375 and 1456 cm^{-1}. The density of amino-functionalized groups on the pore surfaces of MCFs was controlled by the molar ratio of ATES to TEOS from 0 to 2.5 in the initial precursor of post-synthesis procedure. It can be seen from Fig. 4 that the symmetrical bending band at 1505 and 1625 cm^{-1} corresponding to the $-NH_3^+$ group

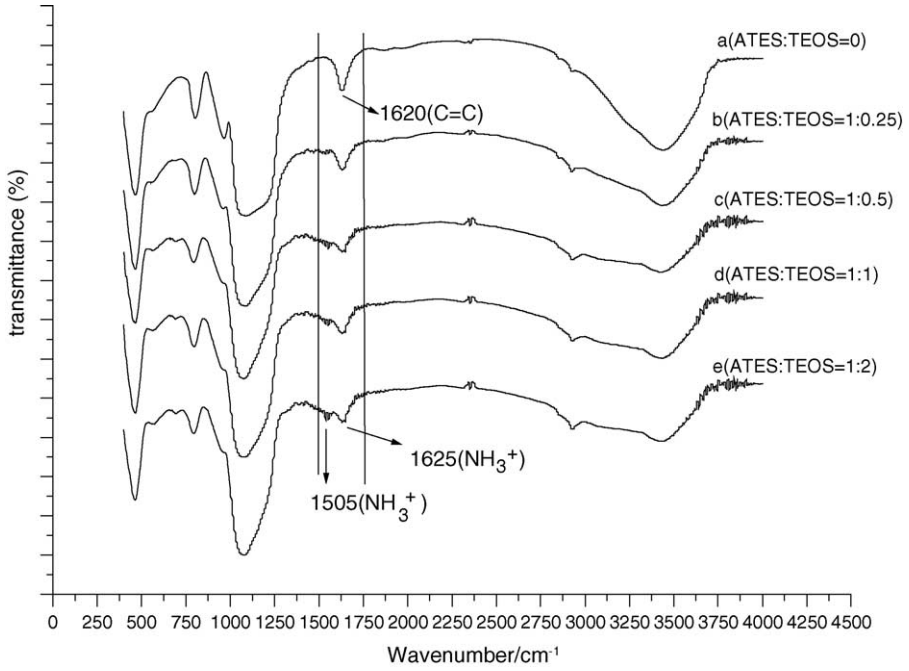


Fig. 4. FTIR spectra of the amino-functionalized mesostructured cellular foam (AF-MCF) materials prepared by microemulsion templating with different molar ratio of TOES: ATES in the initial precursors. (a) 0, (b) 1:0.25, (c) 1:0.5, (d) 1:1 and (e) 1:2.

increases with the molar ratio of ATES to TEOS in the initial precursor of post-synthesis procedure.

3.1. Enzyme immobilization

The immobilization of glucose oxidase with a molecular weights 33,000D onto AF-MCF materials was performed in order to evaluate the adsorption ability of prepared amino-functionalized mesostructured cellular foam (AF-MCF) materials. Fig. 5 shows the amount of GOx immobilized onto AF-MCF materials as a function of the density of amino-functionalized groups on the pore surfaces of MCFs. When the molar ratio of ATES to TEOS was below 0.5, the amounts of enzyme immobilized on AF-MCF materials increase with the increase of the density of amino-functionalized groups on the pore surfaces of MCFs. This phenomenon indicated that the amino groups worked as an active site for enzyme immobilization at low molar ratio of ATES to TEOS. For the molar ratio of ATES to TEOS above 0.5, the adsorption of glucose oxidase remained about equal amounts until 1.25, beyond which the adsorption amount decreases with the increase of the molar ratio of ATES to TEOS. The present of adsorption plateau can be assigned to limited accessibility of the amino groups in the interior of the walls, which depends on pore volume of AF-MCF materials. We believe that high amino concentration will result in a block of mesoporous channel by siloxane groups at post-synthesis procedure. Therefore, the loading of high amino groups on adsorbent causes the decrease of immobilized amounts for enzymes because of some block of mesoporous channel. The optimum molar ratio of ATES to TEOS is 1:0.25. The maximum amount immobilized for GOx here is 210 mg/g AF-MCF (6.36 μmol/g), which is much less than loading of amino groups on AF-MCF materials (2.96×10^3 μmol/g). The result indicated that some of amino groups inside pores are not accessed by Gox. We attribute this to the presence of space obstacle. The fact that the total number of amino groups on support is much higher than the number of enzyme immobilized gives us a considerable implication. The enzyme molecules can covalently couple to the surface of AF-MCF materials by reacting with amino groups because of the amino groups on AF-MCF materials worked as adsorption sites. After enzyme adsorption, the pore volume of AF-MCF materials decreased from 1.38 to 1.32 cm^3/g and the decrease value was about the same volume of enzyme adsorption. It is evidence that the protein molecules can enter mesoporous channels not on the external surface. These results demonstrate the advantage of AF-MCF materials prepared by this method and their potential for the biotechnological application as supports.

3.2. Thermal stability and catalytic activity of immobilized enzymes

In order to investigate the thermal stability of GOx immobilized on LMCF materials, the residual activities of immobilized enzyme and free enzyme after thermal treatment were measured and compared. Fig. 6 shows the residual activity of GOx immobilized on LMCF materials and free GOx, expressed as products of gluconic acid per mg of immobilized enzyme, when the two type enzymes were heated at 55 °C. Although the free enzyme shows the higher activity than that of immobilized enzyme at the initial stage of thermal treatment,

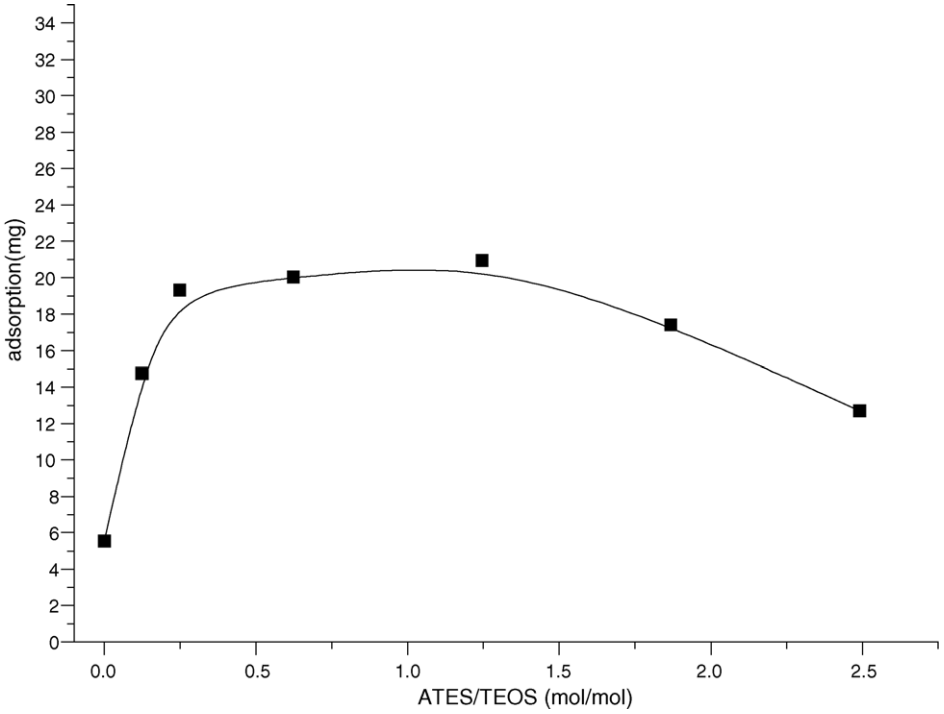


Fig. 5. The amount of enzyme adsorption onto AF-MCF materials as a function of the density of amino-functionalized groups on the pore surfaces of MCF materials.

the activity of the free enzyme decreases quickly with the increase of thermal treatment time, while the activity of immobilized enzyme maintains the relative stable. At 30th min of thermal treatment, the activity of immobilized enzyme becomes the same as that of free enzyme. After 30 min thermal treatment, the activity of immobilized enzyme is higher than that of free enzyme. After 60 min, the free enzyme is almost complete inactivity while immobilized enzyme retained 42% of its original activity (15 min). The activity of immobilized enzyme is still 24% of its original activity after 90 min ther-

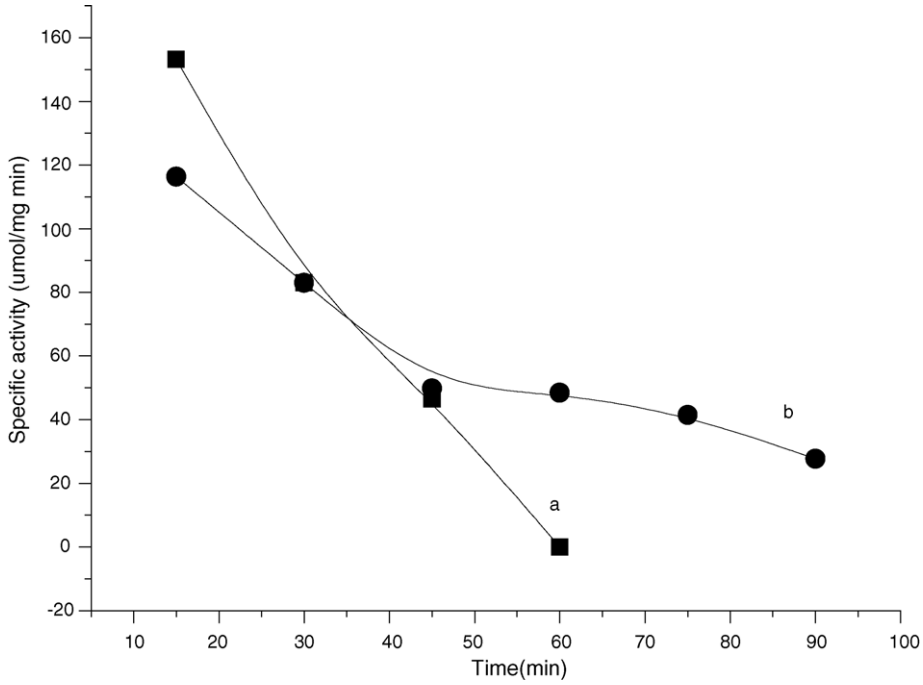


Fig. 6. The thermal stability of GOx immobilized on LMCF materials and free GOx, expressed as a function of the time, when the two type enzymes were heated at 55 °C at phosphate buffer (pH 5.6), (a) free enzyme, (b) GOx immobilized on LMCF materials.

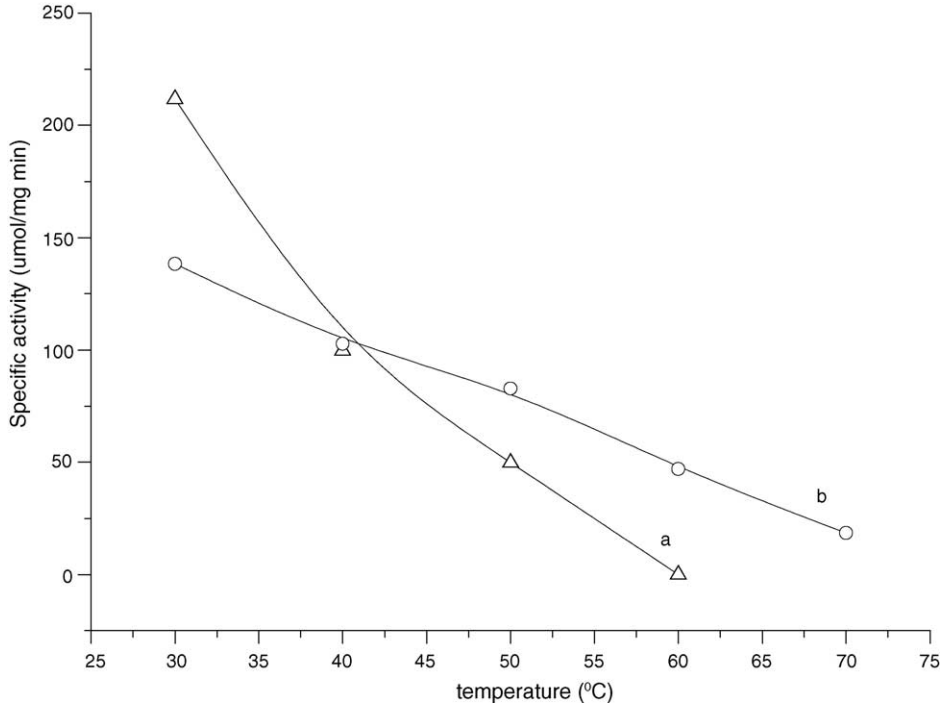


Fig. 7. The catalytic activity vs. temperature on free enzyme and immobilized enzyme at phosphate buffer (pH 5.6) after a 60 min reaction, (a) free enzyme and (b) GOx immobilized on LMCF materials.

mal treatment. Since mesoporous pores of supports can keep enzyme from injuring due to direct exposure environmental change, an immobilized enzyme showed higher thermal stability than that of free enzyme. The activities of GOx immobilized on LMCF materials and free GOx for oxidation of glucose were determined at different temperature ranging from 30 to 70 °C. Fig. 7 showed the activity–temperature curves obtained after 1 h of continuous enzyme catalytic reaction. The plot in Fig. 7a shows that the free enzymes have higher catalytic activity at lower temperature, but its activ-

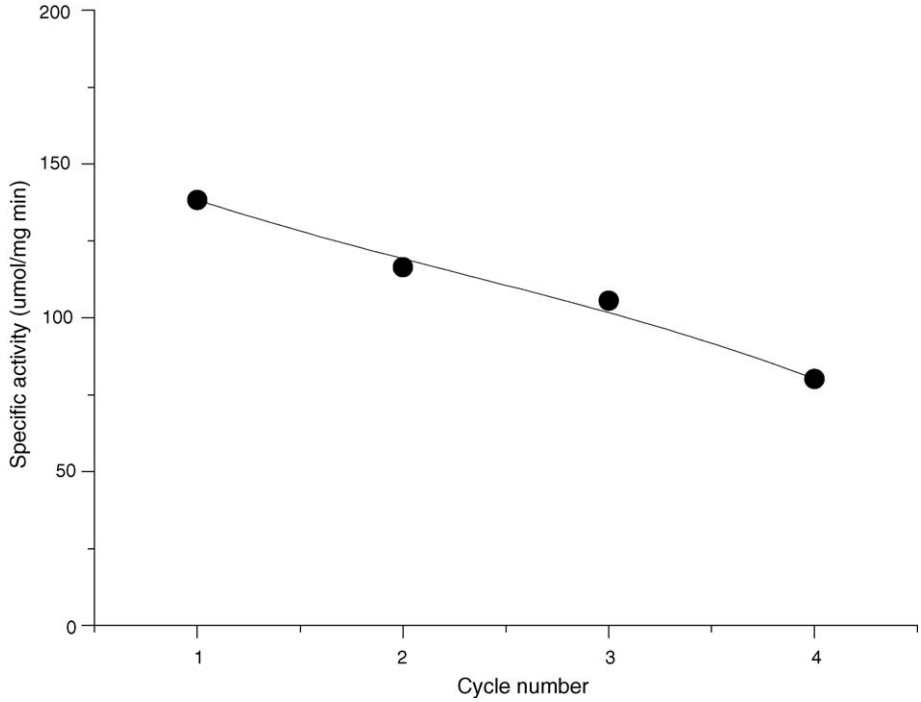


Fig. 8. The catalytic activity of GOx immobilized on LMCF materials vs. the number of recycles use.

ity decrease quickly with the enhancement of temperature. It was also found that the immobilized enzyme activity is 65.3% of free enzymes at 30 °C; however, the immobilized enzyme remained relative stable activity with the increase of temperature. This result is consistent with a previous report [3], in which free enzyme showed higher activities than those of immobilized enzymes at lower temperature due to the inhibition of catalytic activity by amine groups. At 40 °C, the activity of immobilized enzyme is higher than that of free enzyme. For temperature at 60 °C, the free enzyme is almost complete inactivity, while immobilized enzyme retained 34% of its original activity (30 °C). The stable performance of immobilized enzyme at higher temperature can assign to the pore structure and microenvironment of supports, in which the enzymes are protected by wall of mesoporous materials, leading to the increase of stability. The large mesochannels of AF-MCF materials can improve the entrance of biomolecules and decrease the diffusion block of reactants and products. Recovery and re-usability of immobilized enzymes are one of important properties for their applications, although the free enzymes are difficult to recover and re-use. The experimental result indicated the possibility of re-using enzymes immobilized on LMCF materials. Fig. 8 showed that the second cycle is 84.2% of the initial activity and the activity kept 58% of the initial activity after used for four cycles. We performed the leach test of GOx immobilized on LMCF materials and observed less 1% of leaching. We attribute this to the strong interaction between the enzyme molecules and the functional groups on the surface of LMCF materials.

4. Conclusions

Large mesopores cellular foam (LMCFs) materials were synthesized using microemulsion templating in acidic solutions. Amino groups were attached to the surface of LMCFs materials by post-synthesis grafting. The structural and chemical properties of these amino-functionalized porous materials were characterized. These resulting materials showed a high affinity for the immobilization of enzyme molecules (GOx). It is found that the GOx immobilized on AF-MCF materials has the high catalytic activity and thermal stability. The amino-functionalized MCF materials prepared show the advantage as receptors for biomolecules in enzyme catalysis applications where mass transport is often limited by small pore size.

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