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Plasma treatment of multi-walled carbon nanotubes for lipase immobilization

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Abstract−Plasma-modified multiwalled carbon nanotubes (MWNTs) were used as a support to immobilize lipase. The effects of vacuum plasma treatment power, vacuum plasma treatment time, immobilization temperature, immobilization time, and initial protein concentration of the lipase on the amount of lipase immobilized and on the subsequent activity of the immobilized lipase were investigated. The results showed that the adsorption capacity of the plasma-modified MWNTs could reach 0.15 g/g and that the maximal enzyme activity of the immobilized lipase was 520 U/g under optimized conditions. Fourier transform infrared (FTIR) analysis and transmission electron microscopy (TEM) were used to characterize the properties of the plasma-modified MWNTs and plasma-modified MWNTs-lipase, and the results showed that the lipase was successfully immobilized on the plasma-modified MWNTs. Also, the MWNTs-lipase produced an esterification rate of approximately 47% in the synthesis of polyethylene glycol (PEG)-aliphatic esters.

Keywords: Plasma-modified, Multiwalled Carbon Nanotubes, Lipase, Esterification

INTRODUCTION

Lipases have emerged as key enzymes in the rapidly growing field of biotechnology and have proved to be increasingly important biocatalysts for various applications [1]. Owing to their multifaceted properties and huge potential, lipases are used in a wide array of applications in the biomedical sciences, food technology, and fine chemical engineering [2]. To enhance their thermal and operational stabilities and recoverability, lipases are often immobilized on solid supports such as resins [3], sol-gels [4], mesoporous silicas [5], some nanomaterials [6], and so on [7].

Because of their unique size and physical properties, including large surface areas, reduced mass transfer resistance, and effective substance loading, nanometer-sized materials possess many attractive advantages [8,9]. Among the various nanostructured materials, including nanoparticles, nanosheets, nanofibers, and nanotubes, carbon nanotubes (CNTs) have been the focus of much research because of their performance characteristics [10]. The unique structural and physicochemical properties of CNTs make them attractive for applications in many scientific and technological fields such as polymer composites [11,12], electronic structures [13,14], biosensors [15,16], adsorption, and many others [17,18]. Single-walled nanotubes (SWNTs) are attractive for their unique physicochemical properties and larger surface areas [19,20], but MWNTs are desirable because they are simpler to prepare and of lower cost. Therefore, MWNTs are suitable materials as enzyme supports [21,22].

However, it is well known that impurities such as byproduct carbonaceous species and residues from transition metal catalysts used in preparing MWNTs are generated during production of MWNTs [23]. Moreover, due to large draw ratios and strong van der Waals interactions, MWNTs hold tightly together, forming bundles that are insoluble in all organic solvents and aqueous solutions [24]. This lack of solubility and the fact that they are difficult to manipulate in any solvent restricts the overall yield of usable material and interfere with most of the desired properties of MWNTs. MWNTs can undergo functionalization to enhance their dispersibility in various solvents, making them suitable for interacting with other substances. The main approaches being considered for the surface modification of quasi one-dimensional structures can be grouped into three categories: (a) covalent attachment of chemical groups onto the walls of the nanotubes; (b) noncovalent molecule adsorption or the wrapping of various functional molecules onto the conjugated skeleton of MWNTs; and (c) endohedral filling of their inner empty cavities [25]. However, the covalent attachment of chemical groups might destroy the perfect structure of the nanotubes. Also, the main potential disadvantage of noncovalent attachment is that the forces between the wrapping molecules and the MWNTs might be weak [26].

As a simple and convenient method, plasma treatment is a relatively common and important technology for modifying the surfaces of materials before using them in biological evaluation studies [27]. Vacuum plasma treatment can clean or degrease the surfaces of materials, increase the surface energy, and induce surface modification [28]. Therefore, vacuum plasma treatment can be used to readily remove the impurities in prepared MWNTs. Unlike other modification methods, the surface energy of MWNTs after plasma treatment is increased, making them more hydrophilous. At the same time, the surface area of the MWNTs is enlarged.

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The main tasks in designing a perfect immobilized biocatalyst are to choose a suitable carrier, establish appropriate conditions, and select an appropriate enzyme. In the present work, we have chosen MWNTs as the support and Lipozyme CALB L as a soluble lipase [29]. Vacuum plasma treatment was used to modify the MWNTs before lipase immobilization. Then, the effects of reaction conditions on the amount of lipase immobilized were studied. Furthermore, to test the catalytic ability of the immobilized lipase, the plasma-modified MWNTs-lipase was used to catalyze the esterification reaction that synthesizes polyethylene glycol (PEG) aliphatic esters (PEG-laurate, PEG-oleate).

MATERIALS AND METHODS

1. Materials

Lipozyme CALB L was purchased from Novozymes (Denmark). The protein concentration of the Lipozyme CALB L was 10 mg/mL. MWNTs (length of 10-30 μm and outer diameters of 20-40 nm) were purchased from Bo Yu Gao Ke Company Limited, China. Other solvents and chemicals were of analytical grade and used without further purification.

2. Immobilization of Lipases onto MWNTs

MWNT samples were put into the chamber of a vacuum plasma machine. The vacuum pump retained trace amounts of air in the chamber. Under the force of electricity, the residual air could produce plasma. The MWNTs were treated at different vacuum plasma powers, which were controlled by setting the pulse width of the machine. After treating the samples for a certain time, the modified MWNTs were exposed to atmosphere for 1 h.

A phosphate buffer solution (20 mL) containing Lipozyme CALB L was mixed with 50 mg of MWNTs that had been subjected to vacuum plasma pretreatment. The mixture was stirred magnetically for 10-60 min. The enzyme solution and immobilized lipase were separated by filtration. The plasma-modified MWNTs with lipase were dried in a vacuum drying oven. The protein concentrations of the solutions before adsorption and after filtration were measured to determine the amount of protein immobilized.

3. Measurement of Protein Content and Enzymatic Activity

The protein content in the crude enzyme solutions or immobilized enzyme preparations was determined according to the Bradford method, which uses bovine serum albumin as the standard [30]. The assay mixture consisted of 4 mL of Bradford reagent and 1 mL of test solution. The absorbance was read after 3 min standing at 595 nm. The amount of immobilized enzyme on the support (MWNTs or plasma-modified MWNTs) was calculated indirectly from the difference between the amount of enzyme introduced into the reaction mixture and the amount of enzyme in the filtrate after immobilization.

The activity of the immobilized lipase was determined by measuring the release of the acid moiety of an ester by titration with 0.02 M sodium hydroxide solution [31]. A certain amount of plasma-modified MWNTs-lipase was dispersed into a triacetin emulsification solution, which was a pH-stabilized mixture of triacetin (2.0 g), deionized water (50 mL), and pH 7.0 phosphate buffer solution (10 mL). The reaction emulsion was stirred for exactly 30 min at 40 °C. The reaction was then stopped by adding 15.0 mL of a 95% alcohol solution, which inactivated the immobilized lipase. Finally, the reaction was titrated with a 0.02 M sodium hydroxide solution. The volume of sodium hydroxide solution consumed was measured and the activity of the immobilized lipase calculated in the standard way, with one unit of immobilized lipase activity (denoted by U) defined as 1 μmol of acetic acid produced per minute under the assay conditions.

4. Characterization

Analyses of the chemical functional groups of the MWNTs and the plasma-modified MWNTs-lipase were conducted by means of Fourier transform infrared (FTIR) spectroscopy (NEXUS870, USA). Each sample was ground thoroughly with potassium bromide and the resulting powder was pressed to form a transparent pellet using a hydraulic press. FTIR spectra were collected in transmission mode between 500 and 4,000 cm^{-1} at a resolution of 2 cm^{-1}. Transmission electron microscopy (TEM, JEM-200CX, Japan) was used to show the morphology and structure of the MWNTs, the plasma-modified MWNTs, and the immobilized lipase.

5. Lipase-catalyzed Esterification Reaction in a Solvent-free System

12.5 mmol of PEG was gradually added to a 100-mL conical flask that contained 25 mmol of dissolved fatty acids (lauric acid, oleic acid) and 115 mg of plasma-modified MWNTs-lipase (or 1.5 mL Lipozyme CALB L, which has the same protein content as 115 mg of immobilized lipase). The mixture was magnetically stirred for 24 h at 45 °C. The fatty acid content before and after the esterification reaction was measured by titrating with a 0.1 mol/L potassium hydroxide solution. The rate of esterification could be calculated from the change in concentration of the fatty acids.

RESULTS AND DISCUSSION

1. Optimization of Experiment Parameters for Lipase Immobilization

1-1. Effect of Plasma Treatment Power

The effect of vacuum plasma power on the amount of lipase

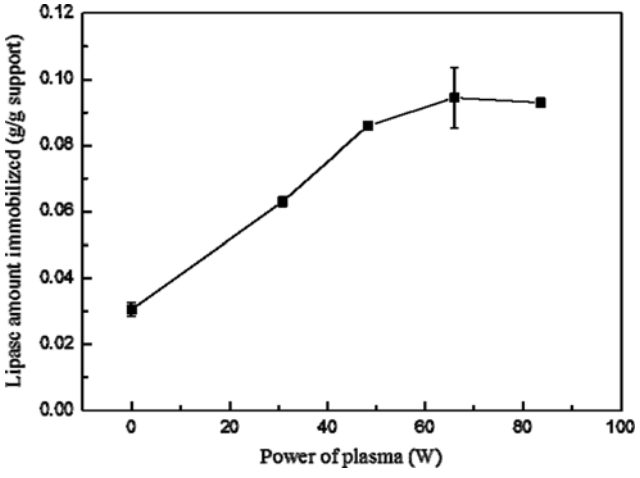

Fig. 1. Effects of vacuum plasma power on lipase amount immobilized. Each of data was assayed in triplicate and the average with standard deviations was measured.

immobilized on the MWNTs was investigated at different intensities from 0 to 83.6 W (Fig. 1). Different from atmospheric plasma, with a relatively low power of electricity, vacuum plasma can produce many radicals, ions, and metastable species depending on the trace amounts of air, which was residual in the current process. The oxygen in the residual air can create some reactive groups on the surfaces of MWNTs, such as peroxides or hydroxides, without changing the entire characteristics of MWNTs. In addition, these reactive groups shift the MWNTs from hydrophobic to hydrophilic [32]. The surface modification of MWNTs using vacuum plasma results in increasing surface energy. Surface energy and surface chemistry changes are particularly important for improving adhesion [28]. We controlled the vacuum plasma power by setting the pulse width of the machine. As compared to raw MWNTs, the plasma-modified MWNTs exhibited better immobilization efficiency because the vacuum plasma treatment readily removed contaminants on the surfaces of the MWNTs. Meanwhile, the surface area of the MWNTs was enlarged and the surface energy increased, improving the enzyme immobilization efficiency of the MWNTs. In general, a higher plasma power can modify the physical surfaces of MWNTs better, thus facilitating adsorption of the soluble lipase.

1-2. Effect of Vacuum Plasma Treatment Time

The effect of vacuum plasma treatment time was studied between 10 and 60 min. As the modified MWNTs became more hydrophilic under longer treatment time, their dispersibility in the phosphate buffer solution improved significantly (data not shown). Fig. 2 shows the effect of treatment time on the amount of lipase immobilized on the MWNTs. Up to 20 min, the lipase amount immobilized increased rapidly. This demonstrated that the plasma treatment time improved the performance of MWNTs over a short period of time, making MWNTs more beneficial for immobilization. After 20 min, the amount of lipase immobilized remained steady. Taking the immobilization efficiency and treatment cost into consideration, the optimal treatment time is 20 min.

1-3. Effect of Immobilization Temperature

The effect of immobilization temperature on the amount of lipase immobilized was measured at different temperatures in the range of 30 °C-70 °C (Fig. 3). As is well known, high temperatures can accelerate the striking force between plasma-modified MWNTs and lipase. However, physical adsorption is an exothermic reaction, and so higher temperatures are not favorable for lipase immobilization on MWNTs. Moreover, elevated temperatures can induce protein denaturation and deactivation [33].

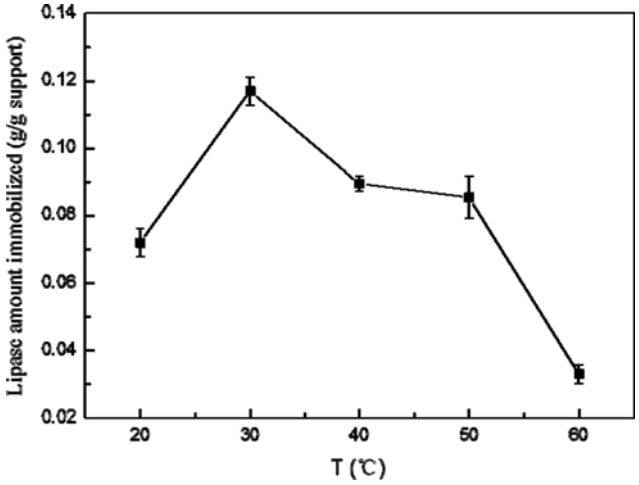

Fig. 3. Effect of immobilizing temperature on lipase amount immobilized. Each of data was assayed in triplicate and the average with standard deviations was measured.

1-4. Effect of Immobilization Time

The effect of the immobilizing time on the amount of lipase immobilized was studied at different reaction times in the range of 10-60 min. During adsorption, two distinct phases could be observed (Fig. 4). In the first phase (up to 20 min), the amount of loaded lipase increased rapidly; in the second phase (from 20 min to 60 min), it slowly approached equilibrium.

1-5. Effect of Protein Concentration

The amount of enzyme loading initially increased linearly with

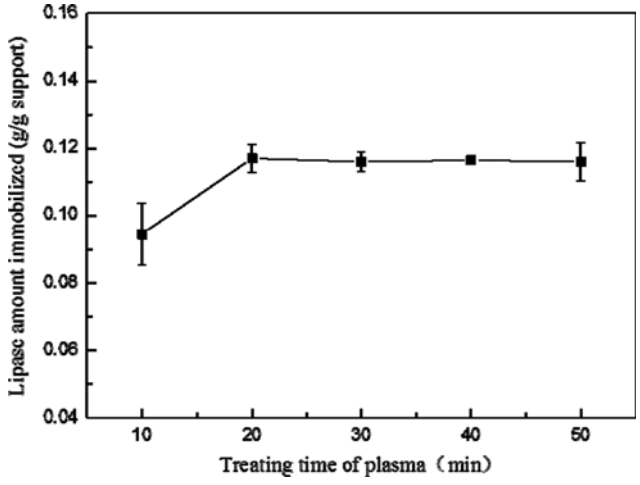

Fig. 2. Effect of vacuum plasma treatment time on lipase amount immobilized. Each of data was assayed in triplicate and the average with standard deviations was measured.

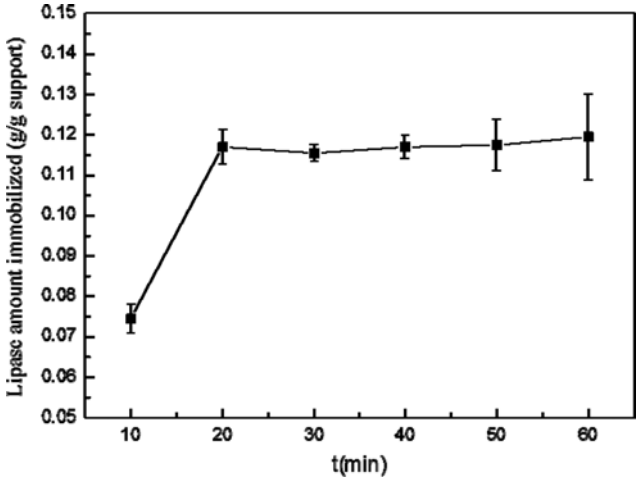

Fig. 4. Effect of immobilizing time on lipase amount immobilized. Each of data was assayed in triplicate and the average with standard deviations was measured.

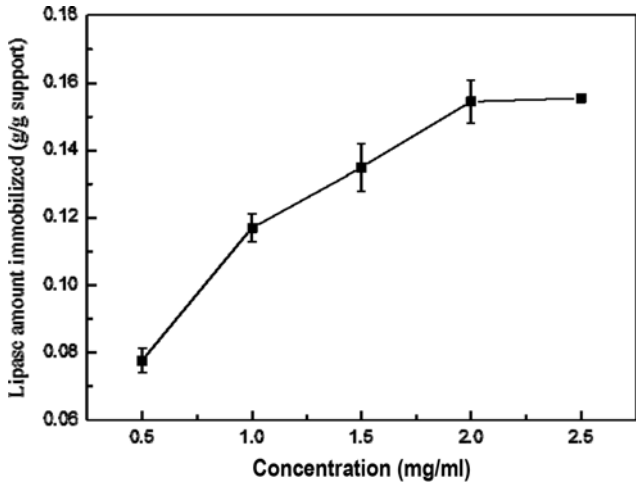

Fig. 5. Effect of protein concentration on lipase amount immobilized. Each of data was assayed in triplicate and the average with standard deviations was measured.

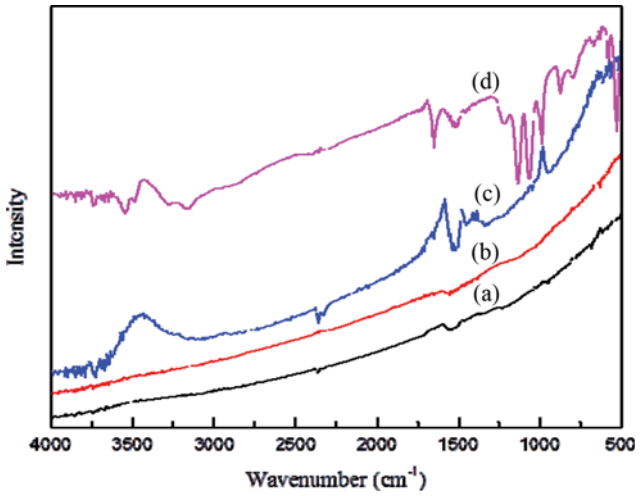

Fig. 6. FTIR spectra of (a) pristine MWNTs, (b) plasma-modified MWNTs, (c) pristine MWNTs-lipase, and (d) plasma-modified MWNTs-lipase.

the initial addition of protein concentration and then became stable when the initial protein reached a certain concentration [34]. The effect of the initial protein concentration on the immobilization was studied at different concentrations in the range of 0.5-2.5 mg/mL. The immobilization experiments were conducted at 30 °C for 20 min with continuous shaking. Fig. 5 shows that the amount of lipase immobilized on the plasma-modified MWNTs increased significantly with increasing protein concentration, reaching a maximum at a lipase protein concentration of 2.0 mg/mL.

The optimized conditions of the immobilization experiment were a plasma power of 74.8 W, a plasma treatment time of 20 min, an immobilizing temperature of 30 °C, an immobilizing time of 20 min, and an initial protein concentration of 2 mg/mL. The results showed that the adsorption capacity of the plasma-modified MWNTs reached 0.15 g/g and that the maximal enzyme activity of the immobilized lipase was 520 U/g under optimized conditions.

2. Characterization of Plasma-modified MWNTs and Immobilized Lipase

The FTIR spectra of the pristine MWNTs, the plasma-modified MWNTs, the pristine MWNTs-lipase, and the plasma-modified MWNTs-lipase are shown in Fig. 6. The pristine MWNTs are not sensitive to FTIR, and so no obvious peak is observed (Fig. 6(a)). This is similar to previous FTIR findings [35]. Because vacuum plasma treatment did not add many functional groups to the surfaces of the MWNTs, there was also no obvious FTIR peak shown in Fig. 6(b). Only a few lipase molecules were immobilized on pristine MWNTs, and thus peaks of the functional groups of lipase are indistinct (Fig. 6(c)). However, it is clear that all charac-

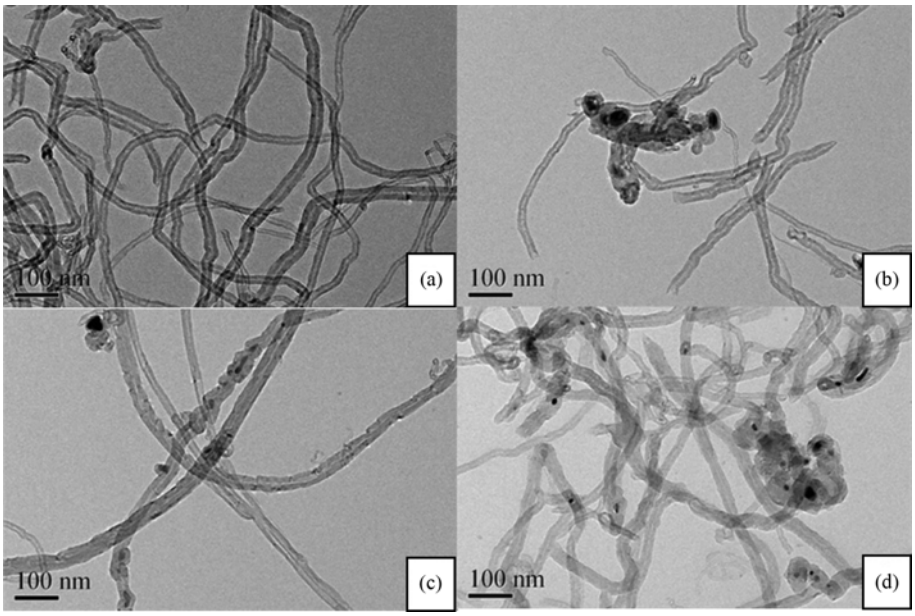

Fig. 7. TEM images of pristine MWNTs (a), plasma-modified MWNTs (b), (c), and MWNTs-lipase (d).

Table 1. Esterification rates of PEG aliphatic esters by different lipases

	Immobilized lipase	Lipozyme CALB L
PEG-laurate	46.85%±2.00%	49.73%±1.84%
PEG-oleate	47.04%±1.82%	50.07%±0.87%

Each of data was assayed in triplicate and the average with standard deviations was measured

teristic bands of the protein are present in the spectrum of the plasma-modified MWNTs-lipase, which undoubtedly confirms that immobilization was successful (Fig. 6(d)). As can be seen in the figure, characteristic peaks for amide I (1,620-1,680 cm^{-1}), amide II (1,480-1,580 cm^{-1}), and amide III (1,225-1,300 cm^{-1}) vibrations are found in the FTIR spectra of the plasma-modified MWNTs-lipase. The two bands in the range of 1,000-1,250 cm^{-1} belong to the C-N stretching vibration. In brief, the FTIR analysis indicated that lipase was likely adsorbed onto MWNTs, as expected.

The size and morphology of the pristine MWNTs, the plasma-modified MWNTs, and the MWNTs-lipase were observed by TEM with the same resolution (Fig. 7). The pristine MWNTs are long and thick (Fig. 7(a)). Because vacuum plasma treatment can induce physical surface modification, some plasma-modified MWNTs were shortened (Fig. 7(b)) and roughened (Fig. 7(c)). Incremental increases in the thicknesses of the MWNT side walls after enzyme immobilization confirmed the presence of the enzyme (Fig. 7(d)). The lipase seemed to cover the entire surface of the MWNTs, as reflected by the evenness of the enzyme coating.

3. Catalytic Activities of Different Lipases

To investigate the catalysis characteristics of plasma-modified MWNTs with immobilized lipases, esterification of the PEG aliphatic esters was carried out. Table 1 shows the esterification rates of PEG aliphatic esters (PEG-laurate, PEG-oleate) by immobilized lipase and Lipozyme CALB L. Plasma-modified MWNTs-lipase produced esterification rates of 46.85% and 47.04% in syntheses incorporating PEG-laurate and PEG-oleate, respectively, values that were slightly less than that of Lipozyme CALB L.

The lipase immobilized on the plasma-modified MWNTs maintained the original activity of Lipozyme CALB L. The immobilization process had almost no impact on the properties of the lipase. Soluble lipase is difficult to separate from the final reaction medium for subsequent catalysis and may contaminate the product. However, with plasma-modified MWNTs-lipase, this problem can be easily solved by filtration. The results showed that plasma treatment is a highly efficient method for modifying MWNTs prior to lipase immobilization.

CONCLUSION

Plasma-modified MWNTs that exhibited superior performance were used as a support to immobilize lipase by physical adsorption. The optimized conditions of the immobilization experiment were a plasma power of 74.8 W, a plasma treatment time of 20 min, and immobilization temperature of 30 °C, an immobilization time of 20 min, and an initial protein concentration of 2 mg/mL. The results showed that the adsorption capacity of the plasma-modified MWNTs reached 0.15 g/g, and the maximal enzyme activity of the immobilized lipase was 520 U/g under the optimized conditions. The FTIR and TEM data further showed that Lipozyme CALB L was successfully adsorbed on the surfaces of the plasma-modified MWNTs. The immobilized lipase produced an esterification rate of approximately 47% in the synthesis of PEG-aliphatic esters.

ACKNOWLEDGEMENTS

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