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Comparison of physical and covalent immobilization of lipase from *Candida antarctica* on polyamine microspheres of alkylamine matrix

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Abstract

Polyamine microspheres (PA-M) prepared using polyethyleneimine as matrix were used for the immobilization of *Candida antarctica* lipase. The isoelectric point of PA-M is 10.6, and the hydrophobicity of PA-M was indicated using naphthalene. Optimization of conditions showed that the maximal loading of lipase on PA-M reached 230.2 mg g^{-1} at pH 9.0 and 35°C. An increased buffer concentration had no effect on the activity of lipase but decreased the amount of lipase adsorbed. Simulation with Langmuir and Freundlich isotherms demonstrated that the adsorption of lipase on PA-M was thermodynamically favorable. Covalent crosslinking of the lipase adsorbed extended the pH range and increased the optimal temperature of the lipase activity. The physically adsorbed lipase (P-lipase) and the covalently immobilized derivative (C-lipase) retained more than 75% and 85% of their initial activity, respectively, after 10 cycles of usage. The half-lives of P-lipase and C-lipase at 50°C were 15.70 and 27.67 times higher than that of the free enzyme, respectively. Compared to P-lipase, covalent immobilization obviously reduced the catalytic efficiency and activation energy of the enzyme.

Keywords: *Covalent immobilization, electrostatic attraction, hydrophobicity, lipase, polyamine microspheres, physical adsorption*

Introduction

Enzyme immobilization is a widely used method to impart desirable features to enzymes (Ansari & Husain 2012; Mateo et al. 2007). Reactions catalyzed by enzymes immobilized on suitable supports have advantages over those catalyzed by soluble enzymes such as ease of separation, reusability, and better stability against inactivation (Bornscheuer 2003). An industrial process should be more economical by using immobilized systems (Hasan et al. 2006; Mateo et al. 2007; Ranganathan et al. 2008).

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are versatile biocatalysts and are widely used in the food, pharmaceutical, and chemical industries (Tan et al. 2010). Various materials have been used in the immobilization of lipase for improving the performance of the enzyme during industrial operation. For examples, alkyl modified diatomite, $CaCO_3$, mesoporous silicate, and activated carbon have been used due to the low cost of inorganic material (Forde et al. 2010; Ghamgui et al. 2007; Ramani et al. 2010; Yang et al. 2009); and glyoxyl agarose, chitosan, dextrans, cyclodextrin, and alginate were used on account of their biocompatibility and biodegradability (Brigda et al. 2007; Monier et al. 2010; Ozmen et al. 2009; Tahir et al. 2009; Mendes et al. 2013).

Lipases are hydrophilic enzymes with hydrophobic regions surrounding the catalytic site (Grochulski et al. 1993). The attachment of lipase to the surface of a support through hydrophobic interaction has been widely adopted as a practical immobilization method (Blanco et al. 2007; Gitlesen et al. 1997; Gunnlaugsdottir et al. 1998; Li et al. 2011). It was also found that hydrophobic interaction between lipase and supports can promote the activity of immobilized lipase (Chen et al. 2012; Dong et al. 2013; Jin et al. 2011; Sorensen et al. 2010; Tao et al. 2014). The adsorption of lipases on hydrophobic

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supports leads to interaction of the hydrophobic areas surrounding the active site, leaving the active site exposed to the reaction medium (Kang et al. 2007; Rodrigues et al. 2013). However, a very high hydrophobicity of the surface has adverse effects on the adsorption of lipase by limiting access of enzyme molecules in aqueous solution to the support (Blanco et al. 2007; Galarneau et al. 2006). Polar agents such as ethanol have been applied to decrease the hydrophobicity of supports to improve the adsorption of lipase (Blanco et al. 2007). Electrostatic attraction is another force driving the adsorption of lipase on supports (Ye et al. 2007; Yong et al. 2008; Salis et al. 2005). Compared to hydrophobic interaction, the force of electrostatic interaction is more stable, tightening the attachment of lipase on the support and reducing leakage during washing and other steps (Kharrat et al. 2011). To take the advantages of both two kinds of interaction, Zheng et al. recently developed a silica particle functionalized with octyl and sulfonic acid groups to immobilize lipase via hydrophobic and strong cation-exchange interaction (Zheng et al. 2012). The immobilized enzyme exhibited remarkable thermal stability and reusability.

PEI, a polycationic polyamine, is a polymer with a branched backbone of two carbons followed by one potentially protonated nitrogen atom. It has the highest cationic density of any synthetic polymer currently available (Kitazoe et al. 2005; Kouisni & Rochefort 2009). PEI has been used to coat the surface of supports to increase the loading of lipase and improve the stability of the enzyme (Cui et al. 2013; Feng et al. 2013). However, the physical coatings of PEI on substrates through electrostatic adsorption are structurally unstable and detach easily in the absence of chemical bonds (Grunlan et al. 2005; Ji et al. 2004). Therefore, covalent grafting of PEI on the substrate has been employed to form stable tethers, avoiding amine leaching (Kassab et al. 2012). Epoxy, aldehyde, and alkyl halide functional groups have been used to react with functional amine groups of PEI for the formation of covalent bonds (Gao et al. 2009; Kassab et al. 2012; Wang et al. 2011). Taking advantage of these covalent reactions, a method called layer-by-layer (LBL) assembly was developed for constructing multilayer films of crosslinked PEI via alternate covalent reactions on substrates such as quartz slides, silicon wafers and aminosilanized glass surface (Hu & Ji 2011; Xia et al. 2011). In recent years, PEI hollow particles have been developed from PEI coating microparticles using a core removal method (Sunintaboon et al. 2006; Tong et al. 2008). However, both t he LBL and the core removal method are time-consuming procedures for obtaining a thin shell of crosslinked PEI and demand sophisticated manipulation of synthesis steps, which greatly limit their application.

In this study, a polyamine microsphere of alkylamine matrix, PA-M, was prepared from PEI via Pickering emulsion polymerization. The microspheres have both a high density of ionizable amino groups and hydrophobic patches available to interact with lipase, which ensure both high enzyme loading and maintenance of the lipase activity. Factors affecting the activity recovery and properties of the immobilized lipase are discussed.

Materials and methods

Materials

Polyethyleneimine (PEI, Mw = 70000 Da; 50 wt% solution in water), lipase from *Candida antarctica* type B (1.68 U/mg protein), was purchased from Sigma-Aldrich Shanghai Trading Co. Ltd., Shanghai, China. Glutaraldehyde (25 wt% solution in water) and liquid paraffin were obtained from Sinopharm Chemical Reagent Co., Ltd., Shanghai, China. Calcium carbonate nanoparticles (40–90 nm in diameter) were supplied by Shanghai Yaohua Nano-tech Co. Ltd., China. Other reagents were of analytical grade and used as received. The lipase was purified using a desalting column (Econo-Pac 10 DG, Bio-Rad) before use. Depending on the desired pH, sodium acetate-acetic acid (pH 4.0–5.0), dipotassium phosphate-potassium dihydrogen phosphate (pH 6.0–8.0), borate (pH 9.0), or glycine-NaOH (pH 10.0–12.0) buffers were used. Molarity of the buffer was 25 mmol L^{-1}.

Preparation of PA-M via Pickering emulsion polymerization

80 ml of PEI solution (20 wt%) was mixed with 120 ml of liquid paraffin and 1.44 g $CaCO_3$ nanoparticles and the mixture was homogenized. 150 ml of 25% glutaraldehyde was then added dropwise into the emulsion at a speed of 0.2 mL min^{-1} using a syringe pump under moderate magnetic stirring. After 15 h of reaction at room temperature, microspheres were collected by filtration and rinsed successively with diethyl ether, isopropyl alcohol, and distilled water. Subsequently, under gentle stirring, the microspheres were reacted with 1 g $NaBH_4$ in 100 ml sodium bicarbonate buffer at pH 10 for 2 h to reduce the imine bonds at room temperature. The product, $CaCO_3$/PA-M composite microspheres, was collected by filtration and rinsed successively with diethyl ether and water. For obtaining polyamine microspheres (PA-M), the $CaCO_3$/PA-M composite

microspheres were soaked in 0.001 mol L^{-1} HCl for 2 h to remove $CaCO_3$ particles. Finally, PA-M were collected by filtration and washed with distilled water until the pH value of the washing water became neutral. Fourier transform infrared spectroscopy analysis of PA-M was performed. IR (KBr) cm^{-1}: 3200–3400 (–N–H stretching), 1550–1650 (–N–H in-plane deformation vibration), 1046 (–C–N stretching), 900–650 (–N–H out-of-plane deformation vibration), 2850–2929 (–C–H stretching), 1465 (–C–H asymmetric bending vibration). Illustration of the preparation procedure of PA-M is demonstrated in Scheme 1.

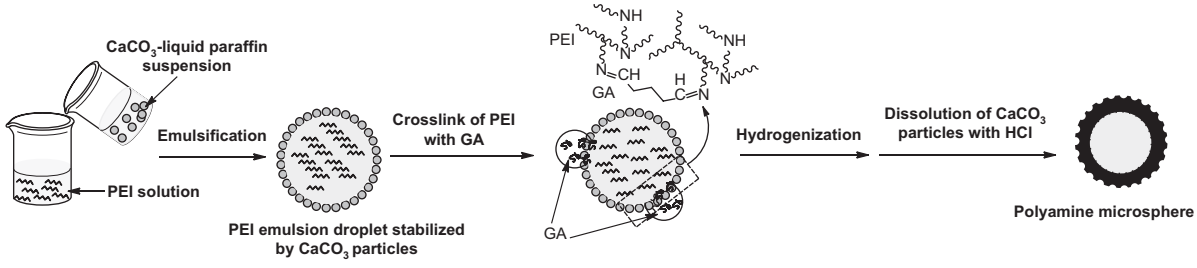
Determination of morphology, zeta potential, hydrophobicity, and thermogravimetry of PA-M

Optical microscopy of PA-M was done using a VHX-1000 Optical microscopy (Keyence Corporation, Japan) equipped with a digital camera (Fujifilm, Japan). The morphological structure of microspheres was examined with an S-4800 scanning electron microscope (Hitachi Ltd., Tokyo, Japan) and the diameter distribution determined by the method of Cohen et al. (Cohen et al. 2011). The average diameter was estimated by counting 200 microspheres. Thermogravimetric (TG) and derivative thermogravimetric (DTG) analysis were performed on a TGA/SDTA851e thermogravimetric analyzer (Mettler-Toledo International Inc., Switzerland). Zeta potentials of PA-M dispersed ultrasonically in the water of different pH were measured using a ZetaPlus zeta potential analyzer (Brookhaven Instruments Corporation, New York, USA) at 25°C.

The hydrophobicity of the surface of PA-M was estimated by measuring its ability to absorb naphthalene (Xiao & Wiesner 2012). 0.05 g microspheres in 10 mL 0.1 M phosphate buffer (pH = 7.5) suspension were added to a 40 mL sample vial and capped with Mininert screw-cap valve (Sigma-Aldrich Co. Ltd.). For each sample, a control vial was set up following the same procedure, while using 10 mL ultrapure water instead of the microsphere suspension, to account for the possible adsorption of naphthalene to the glass and caps. The head space in the vials was approximately 30 mL. Naphthalene-in-acetone stock solutions were then injected with a microsyringe so that the initial naphthalene concentration in the aqueous phase was within the range of 0.05–1.0 mg L^{-1}. The volume fraction of acetone in the aqueous phase in each vial was kept less than 0.002 to avoid possible cosolvent effects. The vials were rotated end-over-end at 20 rpm in the dark at room temperature. After 3 days, the concentration of naphthalene in the head space was analyzed by gas chromatography (GC) using the Shimadzu GC-2010 (Kyoto, Japan) coupled with flame ionization detector. The concentrations of naphthalene in aqueous phase were then calculated based on Henry's Law (with Henry's Law constant for naphthalene at 25°C = 0.0197 [Xiao & Wiesner 2012]) and the concentrations of naphthalene in gaseous phase. All the samples were repeated in triplicate.

Adsorption of lipase by PA-M

In a 50 mL flask with stopper, 0.05 g of PA-M was incubated with 10 mL lipase buffer solution containing 0.1% (v/v) Triton X-100 on a rotary shaker with an agitation speed of 150 rpm at 25°C. The adsorbed lipase was separated by filtration and washed with a buffer solution of the same pH value as the enzyme solution to remove the unbound enzyme. The washing solutions were collected for determination of protein content and enzymatic activity. The amount of enzyme adsorbed was estimated by determining the amount of enzyme in the adsorption solution and the wash buffers. The influence of temperature on the adsorption was investigated over the range of 25–55°C. The data shown in figures and tables are the average values and standard errors of experiments repeated three times. The experimental data on the initial lipase concentration and the amount of lipase adsorbed were fitted into the Langmuir and Freundlich adsorption isotherm model using MicroCal Origins.



Scheme 1. Illustration of the preparation of the polyamine microspheres from PEI via Pickering emulsion polymerization.

The separation factor (R_L) was estimated from the Langmuir adsorption isotherm parameters and used to estimate the affinity between the lipase and PA-M (Weber & Chakravorti 1974).

$$R_L = \frac{1}{1 + K_L C_0} \quad (1)$$

where C_0 is the initial concentration of the lipase (3.5 mg mL^{-1}) and K_L is the Langmuir isotherm constant (mL mg^{-1}). The values of R_L indicate whether the isotherm is unfavorable ($R_L > 1$), linear ($R_L = 1$), favorable ($0 < R_L < 1$), or irreversible ($R_L = 0$).

The treatment of PA-M adsorbed lipase using glutaraldehyde

One gram of lipase adsorbing PA-M was put into a 20 mL solution of 1 wt% glutaraldehyde and incubated for 1 h on a rotary shaker with an agitation speed of 250 rpm at 25°C. Afterwards, the covalently immobilized lipase was collected by filtration and washed several times with distilled water and stored at 4°C.

Protein content and lipase activity determination

The amount of protein in the enzyme solution and in the washing solution was measured by the Lowry method (Lowry et al. 1951).

The enzymatic activities of free and immobilized lipases were measured by detection of the *p*-nitrophenol released by hydrolysis of *p*-nitrophenyl palmitate (*p*-NPP) (Chiou & Wu 2004). 0.5 g of *p*-NPP dissolved in 100 mL of ethanol was used as substrate. The increase in absorbance at 410 nm caused by the release of *p*-nitrophenol in the hydrolysis of *p*-NPP was measured spectrophotometrically. Lipase was added to a mixture of 1 mL of 0.5% (w/v) *p*-NPP solution and 1 mL of 25 mmol L^{-1} phosphate buffer (pH 7.5) at 35°C. After 5 min incubation, the reaction was terminated by adding 2 mL of 0.5 N Na_2CO_3 followed by centrifuging for 10 min (10,000 rpm). The supernatant of 0.5 mL was diluted 10-fold with distilled water, and measured at 410 nm using a Shimadzu 1240 UV-vis spectrophotometer. One unit (U) of lipase activity was defined as the amount of enzyme which released 1 mmol *p*-nitrophenol per minute under the experimental conditions. The relative activity (%) was the ratio between the activity of every sample and the maximum activity of sample. The specific activity (U·mg^{-1} of protein) was calculated by dividing the enzyme activity by the protein content.

Influence of temperature and pH on the activity of lipase

The optimum operating pH and temperature for free and immobilized lipases were determined by measuring the activity of the lipase over a temperature range of 20–60°C and pH from 6.0 to 11.0. The maximum activity of each set was taken as 100%. The relative activity (%) was calculated by comparing the activity of every sample with the maximum activity.

Thermal stability and reusability of immobilized lipase

Reusabilities of the immobilized derivatives during the hydrolysis of olive oil were examined by measuring the residual activities of immobilized enzymes. The conditions of hydrolysis reaction were based on methods described previously (Abramic et al. 1999; Cho & Rhee 1993; Liu et al. 2005). 0.05 g immobilized lipase or 15 mg free enzyme was allowed to react with a mixture of 4 mL olive oil emulsion and 6 mL phosphate buffer (pH 7.5) in 25 mL flasks with stoppers on a rotary shaker at an agitation speed of 150 rpm at 35°C. After each run the immobilized derivatives were separated by filtration, washed with the phosphate buffer of pH 7.5, and then reintroduced into another fresh reaction medium. The olive oil emulsion was prepared by emulsifying 50 mL olive oil in 150 mL distilled water containing 4 wt% PVA (Yang et al. 2010).

The thermal stabilities of free and immobilized lipases were tested at 25°C and 50°C in 25 mmol L^{-1} phosphate buffer of pH 7.5. The reaction mixtures were transferred into an ice bath every hour and the residual activities measured. The initial activity of enzyme was assigned as 100%. The thermal inactivation constant (k_d) and half-life ($t_{1/2}$) for the lipases were calculated using Eqs. (2) and (3), respectively (Cabrera-Padilla et al. 2012; Olusesan et al. 2011).

$$\ln\left(\frac{A_t}{A_0}\right) = -k_d t \quad (2)$$

$$t_{1/2} = \frac{\ln 2}{k_d} \quad (3)$$

where A_t is the residual activity after heat treatment for a period of incubation (U), A_0 is the initial enzyme activity (U), k_d is the inactivation constant (h^{-1}), and $t_{1/2}$ is the half-life (h).

Determination of physicochemical parameters of free and immobilized lipases

The Michaelis–Menten kinetic parameters of the free and immobilized lipases were determined from

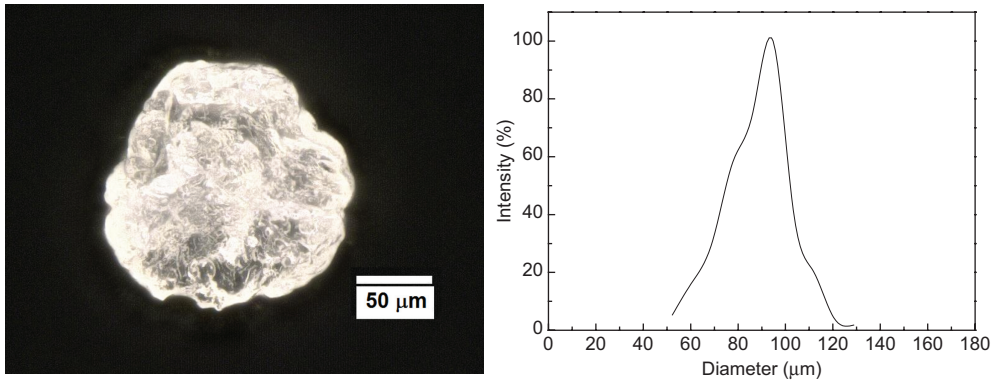


Figure 1. Digital photograph and diameter distribution plot of PA-M.

a Lineweaver–Burke plot. The initial rates of reaction were measured in 25 mmol L^{-1} phosphate buffer (pH 7.5) at 35°C. The catalytic efficiency (η) and the value of turnover number (k_{cat}) were calculated from the following equation:

$$\eta = \frac{k_{cat}}{K_m} = \frac{V_m}{[E]_t K_m} \quad (4)$$

where $[E]_t$ is the enzyme concentration and V_m is the maximal velocity.

The activation energies (*Ea*) of the native lipase and its immobilized derivatives were calculated according to the Arrhenius law.

Results and discussion

Morphology, TG analysis, surface charge and hydrophobicity of PA-M

Pickering emulsions are more stable than ordinary emulsions as they use fine solid powders as the emulsifier (Chen et al. 2010; Kralchevsky & Nagayama 2000). In this study, PEI was cross-linked with glutaraldehyde in liquid paraffin emulsion stabilized with $CaCO_3$ particles, initially producing $CaCO_3$/PA-M composite microspheres. After reduction of imine linkages and removal of the stabilizer, polyamine microspheres PA-M were obtained, which appeared translucent under the microscope (Figure 1). The diameter of PA-M ranged from 48.08 to 132.21 μm, with an average value of 88.25 μm. Scanning electron microscopy images of PA-M are shown in Figure 2. The surface of PA-M was rough and small pores were recognizable on the surface. The TG analysis of PA-M is recorded in Figure 3. The total weight loss of the microsphere was 83.3% from 50°C to 530°C. According to the DTG curves, PA-M shows an initial mass loss over the range from 50°C to 100°C, due to loss of adsorbed water. Corresponding to an obvious endothermic peak in the range from 132.5°C to 204°C, the microsphere lost about 6.5% of its weight, possibly owing to evaporation of small compounds such as residual glutaraldehyde. The maximal decomposition rate of PA-M was found at 440°C and the temperature for total decomposition of PA-M was 530°C.

Investigation of the surface charge on PA-M at different pH showed that the isoelectric point (pI) of the microspheres was 10.6 (Figure 4A). Thus, the

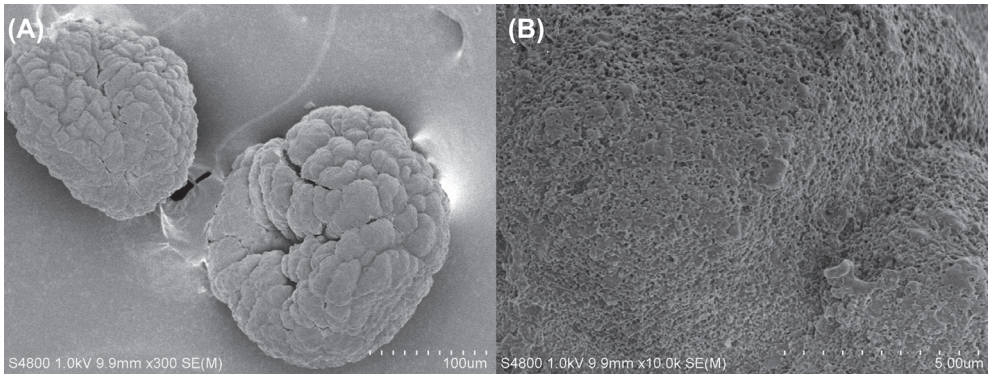


Figure 2. SEM images of PA-M.

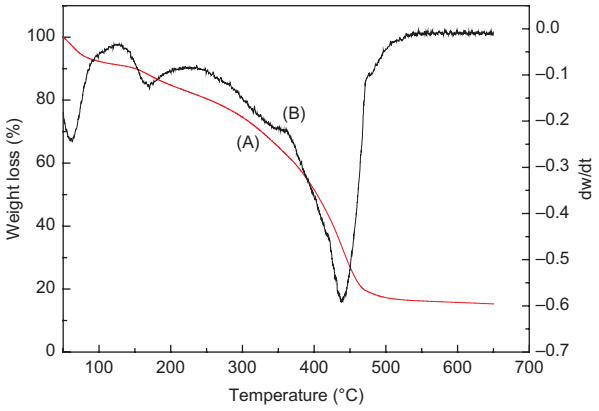


Figure 3. TG (A) and DTG (B) thermogram of PA-M.

surface charge of the microsphere is positive when pH < 10.6 and negative when pH > 10.6. Considering the isoelectric point of the lipase is 6.0, the enzyme should be adsorbed by PA-M through electrostatic interactions in the pH range of 6.0–10.6. The surface hydrophobicity of microspheres was probed using naphthalene as an indicator (Xiao et al. 2012). According to Figure 4B, the amount of naphthalene adsorbed by PA-M increased with the concentration of naphthalene. Clearly, there were hydrophobic patches on the surface of PA-M, which could interact with the hydrophobic surface of lipase (Derewenda et al. 1992), promoting adsorption of the lipase on PA-M. Interaction of PA-M with the hydrophobic lid covering the active center of lipase should facilitate exposure of the active site to the reaction medium, resulting in the activation of enzyme (Kang et al. 2007; Rodrigues et al. 2013).

Physical adsorption and covalent immobilization of lipase on PA-M

The forces driving the adsorption of proteins on surfaces include electrostatic attraction, hydrophobic interactions, and hydrogen binding. The roles played by different driving forces during the adsorption process depend on the specific characteristics of enzyme and the support surface involved. Changes in the pH, temperature, initial concentration of lipase, ionic strength of adsorption medium and the incubation time on the adsorption of lipase by PA-M were investigated and the results are shown in Table I. When the pH of the solution was 10.6 (pI of PA-M), the surface charge of PA-M would be reduced to a minimum, and electrostatic interaction between the lipase and the microsphere decreased to its lowest level (Salis et al. 2005). The adsorption of the lipase on PA-M (65.9%) in this situation would be mainly driven by hydrophobic interaction. When 6.0 < pH < 10.6, lipase and PA-M had opposite charges. The adsorption in this case would be driven by mixed modes of electrostatic and hydrophobic interaction, resulting in the highest yield of lipase adsorption (86.2%) at pH 9.0. When the adsorption was carried out at pH < 6.0 and pH > 10.6, both the lipase and PA-M would have the same charge. Electrostatic repulsive forces should limit the adsorption of lipase on PA-M. However, the adsorption of lipase on PA-M at pH 5.0 and 12.0 still reached 55.78% and 13.7%. Presumably, adsorption in this case is driven by hydrophobic interaction overcoming electrostatic repulsive forces between proteins and materials having the same charge (Andersson & Hatti-Kaul 2000). However, the buffering capacity

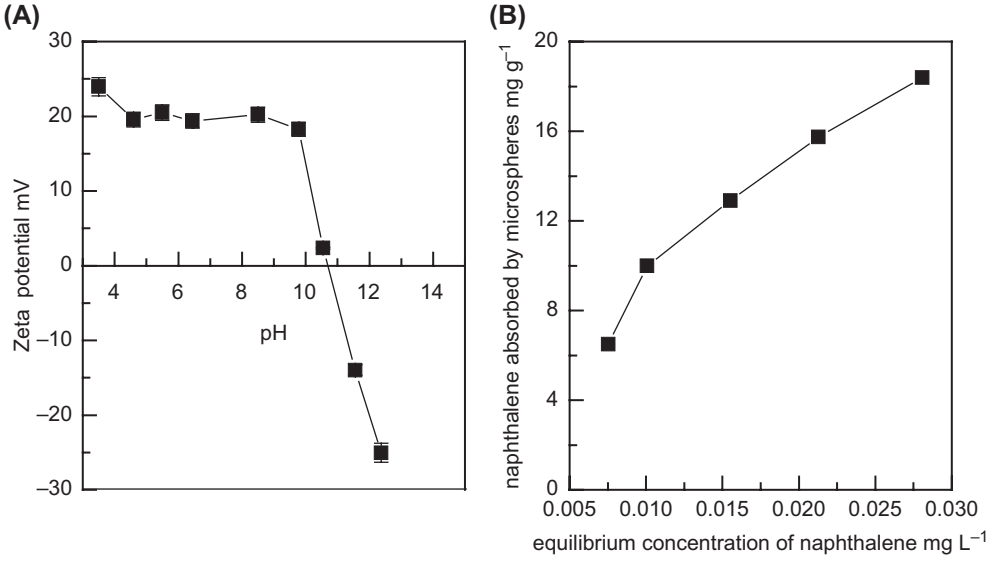


Figure 4. Zeta potential of PA-M dispersed in water of different pH (A) and the amount of naphthalene adsorbed by PA-M at various concentration of naphthalene (B).

Table I. Effects of pH, temperature, initial concentration of lipase, concentration of buffer solution and incubation time on the adsorption of lipase on PA-M.

	Lipase adsorbed (%)	Specific activity (U mg protein $^{-1}$)	Relative activity of lipase (%)
pH*			
5	55.78 ± 3.18	0.79 ± 0.03	64.75 ± 3.79
6	75.4 ± 3.57	0.87 ± 0.03	71.31 ± 3.44
7.5	80.5 ± 3.80	1.05 ± 0.02	86.06 ± 1.90
9	86.2 ± 3.92	0.97 ± 0.04	79.51 ± 4.12
10.6	65.9 ± 3.41	0.66 ± 0.02	54.10 ± 3.03
12	13.7 ± 0.61	0.59 ± 0.01	48.36 ± 1.69
Temperature (°C)*			
25	72.9 ± 2.81	0.94 ± 0.03	77.05 ± 3.19
35	85 ± 2.89	1.08 ± 0.04	88.52 ± 3.70
45	86.1 ± 2.93	1.03 ± 0.04	84.42 ± 3.88
55	87.4 ± 3.03	0.87 ± 0.03	71.31 ± 3.45
Concentration of buffer solution (mmol mL^{-1})*			
25	84.3 ± 4.22	1.05 ± 0.04	86.06 ± 3.85
50	83.29 ± 4.04	1.04 ± 0.03	85.24 ± 2.88
75	77.89 ± 3.62	1.06 ± 0.03	86.88 ± 2.83
100	72.07 ± 3.27	1.04 ± 0.02	85.24 ± 1.92
150	68.03 ± 3.03	1.05 ± 0.04	86.06 ± 3.85
200	65.92 ± 2.91	1.07 ± 0.03	87.70 ± 2.80
250	63.90 ± 2.54	1.06 ± 0.03	86.88 ± 2.83
Incubation time (min)*			
10	56.6 ± 2.83	1.17 ± 0.04	96.13 ± 3.42
20	75.8 ± 3.79	1.10 ± 0.04	90.16 ± 3.64
40	80.4 ± 4.02	1.04 ± 0.03	85.15 ± 2.88
60	82.8 ± 4.00	1.05 ± 0.04	86.06 ± 3.81
80	83.1 ± 4.15	1.06 ± 0.04	87.00 ± 3.77
100	84.39 ± 4.21	1.07 ± 0.04	87.90 ± 3.74
120	78.29 ± 3.91	0.99 ± 0.02	81.49 ± 2.02
140	83.1 ± 4.15	1.06 ± 0.03	87.00 ± 3.44
160	79.1 ± 3.95	0.99 ± 0.04	81.48 ± 4.04
Initial concentration of lipase (mg mL^{-1})	Lipase adsorbed (mg g^{-1} PA-M)	Specific activity (U mg protein^{-1})	Relative activity of lipase (%)
0.41	77.4 ± 3.25	0.86 ± 0.02	70.49 ± 2.32
0.82	144.4 ± 6.06	0.93 ± 0.03	76.23 ± 3.22
1.08	183.2 ± 7.69	1.05 ± 0.04	86.06 ± 3.81
1.91	210 ± 8.82	1.11 ± 0.05	90.98 ± 3.82
2.6	227 ± 9.53	1.20 ± 0.05	98.36 ± 4.12
3.56	230 ± 9.66	1.22 ± 0.05	100 ± 4.20

*The concentration of lipase in solution used was 1.08 mg mL^{-1}.

of the PEI matrix can also offset the influence of pH variation, promoting adsorption at unfavorable pH values. Although the specific activity of lipase adsorbed at pH 7.5 was 1.08 times higher than that of lipase adsorbed at pH 9.0, 9.0 was chosen as the optimal pH for the adsorption of lipase by PA-M due to the higher enzyme loading.

Investigation of the influence of temperature on the adsorption of lipase on PA-M at pH 9.0 (Table I) showed a significant rise in adsorption with a temperature increase from 25°C to 35°C. Variation in temperature can have an important influence on the conformation and activity of proteins (Dill 1990). Elevated temperature can expose the inner hydrophobic core of a folded protein, resulting in increased hydrophobic interaction (Hjerten 1973). According to the results in Table I, the differences in lipase adsorption was not statistically significant in the window of temperature from 35 to 55°C. We surmise that the adsorption of lipase in this range of temperature was controlled by an exothermic process (Duff & Kumar 2009). Since the maximal value of specific activity of the adsorbed lipase was obtained at 35°C, this temperature was chosen as the optimal temperature for the adsorption of lipase by PA-M in the following studies.

The ionic strength of the adsorption medium is another important factor affecting protein adsorption

on supports (Vieira et al. 2011). An increase in salt concentration can reduce the polarity of the solution and also partially decrease the surrounding water structure on the surface of the absorbents and protein, promoting hydrophobic interaction of protein with adsorbents (Chen et al. 2003). In contrast, an increase in ionic strength leads to decreased electrostatic adsorption (Pei et al. 2001), because a competition between protein and charged molecules in the buffer solution occurs during the electrostatic adsorption process. The influence of different salt concentrations on lipase loading on PA-M is shown in Table I. Lipase adsorption decreased with an increasing salt concentration. Although high ionic strength can weaken the electrostatic interaction between lipase and PA-M, the loading of enzyme in 250 mmol L^{-1} buffer still reached 179.4 mg g^{-1}, which was about 78.8% of the maximum loading. Thus, owing to the increased hydrophobic interaction in high salt concentration solution (Chen et al. 2003), PA-M can retain a relative high affinity for lipase in an adsorption medium of high ionic strength. Although ions interfered with the adsorption of lipase, variation in the concentration of buffer solution showed no effects on the activity of adsorbed lipase. This result is similar to that reported by Lee et al. (2010), where the activities of immobilized *C. rugosa* and *R. oryzae* lipases were not influenced by the variation of ion strength when the concentration of buffer solution was less than 250 mmol L^{-1}.

Investigation of the incubation time of lipase adsorption showed that the loading almost reached equilibrium within 40 min at pH 9.0 and 35°C. The maximum loading of lipase, about 230.2 mg g^{-1} PA-M, was obtained at an initial concentration of 3.5 mg mL^{-1} lipase in solution. This loading is approximately equal to that of *Candida antarctica* lipase adsorption on functionalized mesoporous silica (Blanco et al. 2007) and of lipase adsorption on polypropylene powder (Gitlesen et al. 1997) but is higher than that of PEI-coated supports (Cui et al. 2013; Feng et al. 2013; Ondul et al. 2012). Compared to the PEI-coated supports, PA-M of crosslinked PEI matrix offers a higher density of amino groups for interaction with the enzyme.

Langmuir model and Freundlich isotherm were compared in the evaluation of the adsorption process of lipase on PA-M as a unit operation. Some model parameters were determined by nonlinear regression and shown in Table II. Comparing the simulation based on these two models showed that the Langmuir equation fitted the experimental data best. The theoretical maximum adsorption capacity of PA-M, 253.2 mg g^{-1}, is higher than the experimental value of maximal loading. According to the results in Table II, the value of the separation factor R_L was calculated as 0.013, which is greater than 0 but less than 1, suggesting that a Langmuir isotherm was favorable. $1/n$ is a Freundlich constant, indicating the type of isotherm. When $0 < 1/n < 1$, adsorption is favorable; $1/n = 1$, irreversible; $1/n > 1$, unfavorable (Vasiliu et al. 2011). As shown in Table II. The value of $1/n$ indicated that the adsorption of lipase by PA-M was favorable.

Table II. Kinetic constant of Langmuir and Freundlich isotherms of lipase adsorption on PA-M.

Langmuir model				Freundlich model		
q_m	K_L	R^2	R_L	K_f	$1/n$	R^2
253.2	21.2	0.9939	0.013	215.3	0.215	0.8592

Lipase concentration range: 0.41–3.5 mg mL^{-1}; incubation time: 40 min; temperature: 35°C; buffer: 25 mmol L^{-1} borate, pH value of buffer was 9.0.
Where q_m is the maximum adsorption capacity of PA-M for lipase (mg g^{-1}); K_L is the Langmuir isotherm constant(mL mg^{-1}); R_L is the separation factor; K_f the Freundlich constant and n is the Freundlich exponent.

Because of its commercial availability, low cost and high reactivity, glutaraldehyde has been considered as one of the most effective protein crosslinking reagents (Migneault et al. 2004; Richards & Knowles 1968). The aldehyde groups of glutaraldehyde can react rapidly with amine groups at around neutral pH (Okuda et al. 1991) and is more efficient than other aldehydes in generating chemically, biologically, and thermally stable crosslinks (Fernandez-Lafuente et al. 1995; Hwang et al. 2004; Nimni et al. 1987). Hence, glutaraldehyde remains the reagent of choice for crosslinking, although many reagents and newer methods are available (Govardhan 1999). PA-M carries a high density of amine groups that can not only be used for anionic binding of lipase but also can be used as the basis for cross-linking the enzyme with glutaraldehyde. However, glutaraldehyde can cause denaturation of immobilized enzymes (Romdhane et al. 2011). A low concentration of glutaraldehyde is favorable for high activity recovery, while a high concentration of glutaraldehyde tends to inactivate the enzyme (Bhandari et al. 2009; Pan et al. 2011; Park et al. 2002; Wang et al. 2008). Therefore, the effects of glutaraldehyde treatment on the activity of lipase adsorbed on PA-M were inves-

Table III. Deactivation constants (K_d) and half-life ($t_{1/2}$) values for the free lipase and the immobilized derivatives.

	K_d (h^{-1})		$t_{1/2}$ (h)	
	25°C	50°C	25°C	50°C
Native enzyme	0.0469	0.401	14.77	1.73
P-lipase	0.0122	0.0255	56.8	27.17
C-lipase	0.0105	0.0135	66	51.33

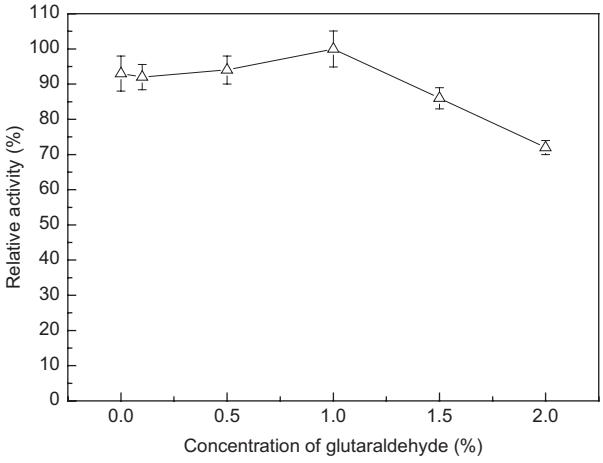


Figure 5. Effect of the amount of glutaraldehyde addition on the activity of lipase.

tigated (Figure 5). The activity of immobilized lipase reached its highest value when 1% glutaraldehyde was added. Any further increase in glutaraldehyde concentration had a negative effect on the lipase activity.

After adsorption and crosslinking, the specific activity of the physically adsorbed lipase (P-lipase) and the covalently immobilized derivative (C-lipase) was determined as 1.22 U mg protein^{-1} and 1.31 U mg protein^{-1} separately, which is slightly less than that of the native enzyme (1.68 U mg protein $^{-1}$).

Optimum pH and temperature for the operation of lipase

The activities of the native and immobilized lipase under different pH conditions are shown in Figure 6. The pH value (pH 7.5) for optimum activity was not changed by physical adsorption. However, the pH range for attaining 90% activity of lipase was extended from 7.2–8.0 (free enzyme) to pH 6.7–8.6 (P-lipase) and pH 6.4–8.7 (C-lipase). This increased adaptability of lipase to variation in pH after immobilization may be due to the buffering capacity of PEI matrix of PA-M (Akinc et al. 2005).

Figure 6B demonstrates the temperature-activity profiles of the native lipase and the immobilized derivatives under the optimum pH conditions. The optimum temperature for the activity of lipase increased from 35°C (the free enzyme) to 40°C (P-lipase) and 40–45°C (C-lipase). Thus, the optimum temperature for activity was increased by immobilization. This result is similar to that where *C. antarctica* lipase was immobilized on cotton terry cloth fibrils coated with PEI (Ondul et al. 2012). Running reactions at elevated temperature are advantageous because of the higher diffusion rate, lower substrate viscosities, and increased reactant solubilities (Hasan et al. 2006).

Thermal stability and reusability

Immobilization of an enzyme to a support often limits the freedom of enzyme molecules to undergo drastic conformational changes, increasing the stability of enzyme towards denaturation. Figure 7A shows that the thermal stability of lipase was increased by immobilization. Incubating at 25°C for 8 h, the free lipase retains about 72.5% of its initial activity, while both P-lipase and C-lipase retain more than 90% of their initial activities. When the incubations were conducted at 50°C, the native lipase lost almost all of its activity, while P-lipase and C-lipase retained about 79.5% and 90% of their initial activity, respectively. Thus, *Candida antarctica* lipase showed a better thermal stability

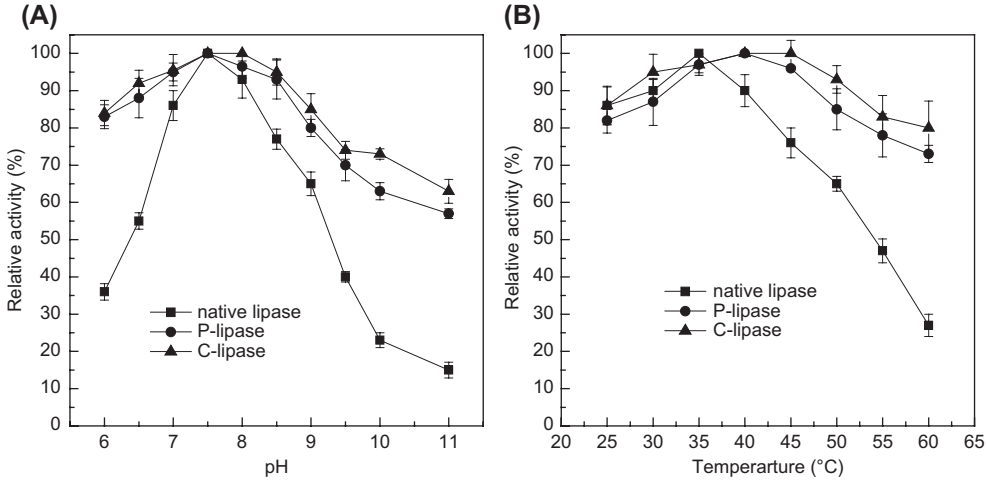


Figure 6. pH-activity (A) and temperature-activity (B) profiles of the native lipase, P-lipase and C-lipase, respectively.

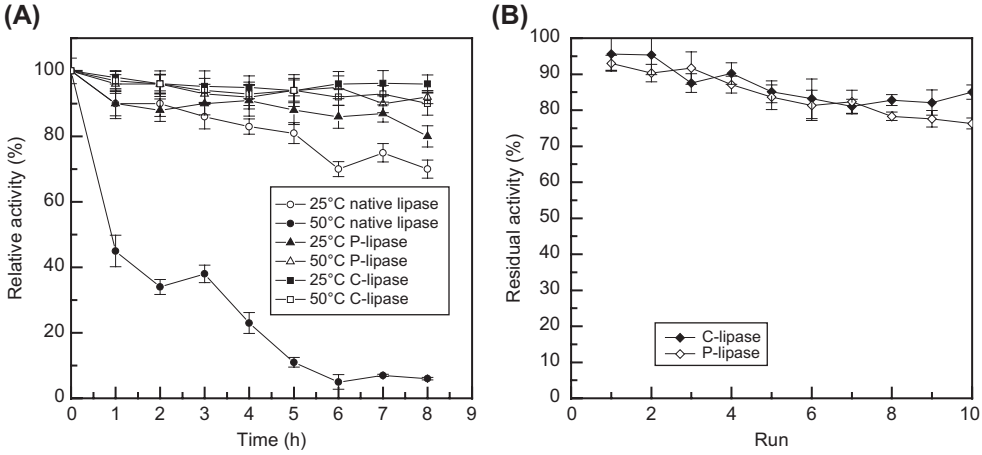


Figure 7. Thermal stability of the native and immobilized lipase (A) and reusability of the immobilized derivatives (B).

on PA-M than that on octyl silica (Blanco et al. 2004). Electrostatic attraction and hydrophobic interactions between PA-M and lipase comprised the main driving forces promoting multipoint interaction between PA-M and enzyme, enforcing conformational stability of the enzyme. Since covalent linkages are more stable than physical interactions, C-lipase showed a higher stability than P-lipase. The half-life values of the free enzyme, P-lipase and C-lipase at 25°C and 50°C are shown in Table III. At 25°C, the $t_{1/2}$ of the free enzyme was approximately 26% and 22.38% of that of P-lipase and C-lipase respectively. At 50°C, the $t_{1/2}$ of P-lipase and C-lipase was 15.70 and 27.67 times higher than that of free enzyme. Thus, the thermal stability of the *Candida antarctica* lipase at the high temperature was significantly improved upon immobilization. The enhanced hydrophobic interactions as temperature increased could increase the thermal stability of immobilized lipase at higher temperature (Cabrera-Padilla et al. 2012). Based on the results of Table III, the values of K_d decreased from 0.0469 h^{-1} (25°C) and 0.401 h^{-1} (50°C) for native enzyme to 0.0122 h^{-1} (25°C) and 0.0255 h^{-1} (50°C) for P-lipase, to 0.0105 h^{-1} (25°C) and 0.0135 h^{-1} (50°C) for C-lipase, respectively. The reduced value of K_d indicates the increased stability of enzyme as a result of immobilization (Olusesan et al. 2011).

The reuse of an enzyme constitutes the main process advantage of biocatalyst immobilization. The reuse of an immobilized enzyme permits simplification of the design of the reactor and the control of the reaction (Kharrat et al. 2011). The investigation showed that P-lipase retained about 75% of its initial activity at the end of 10 rounds of recycling (Figure 7B). The physical adsorbed P-lipase was quite stable during recycling, due to the help of the multipoint electrostatic and hydrophobic interaction. Compared to P-lipase, C-lipase was more stable and retained more than 85% of its initial activity after 10 runs. Covalent linkages are more stable than electrostatic and hydrophobic interactions, limiting configurational changes of enzyme molecules during operation and reducing leakage of the enzyme during recycling.

Kinetic constants of free and immobilized lipase

The value of the kinetic constant, K_m, can reflect the affinity of enzymes to substrates. A low value of K_m represents a high affinity between enzymes and substrates. Kinetic constants of the free and immobilized lipase were determined by using olive oil emulsion as the substrate. The K_m and K_m^{app} for the free lipase, P-lipase and C-lipase were 5.23, 6.79 and 8.76 μmol L^{-1} respectively. The increased value of the Michaelis–Menten constants indicates that the accessibility of substrate to the active sites of the immobilized enzyme molecules was decreased due to the steric hindrance caused by contacting with the support surface (Kang et al. 2005). The values of V_m^{app} for P-lipase and C-lipase were 65.76 and 66.15 μmol L^{-1} min^{-1}, which are respectively 1.19 times and 1.2 times higher than the V_m of the free enzyme, at 54.95 μmol L^{-1} min^{-1}. Since the molecular mass of lipase from *Candida antarctica* type B is 33 kDa (Uppenberg et al. 1995), the catalytic efficiency (η) of the enzyme was calculated as 0.0289 L μmol^{-1} min^{-1} for the native lipase, 0.0265 L μmol^{-1} min^{-1} for P-lipase and 0.0234 L μmol^{-1} min^{-1} for C-lipase. Thus, the catalytic efficiency of lipase reduced about 8.3% (P-lipase) and 19% (C-lipase) after immobilization. This reduction may be caused by steric hindrance of the hard surface of microsphere impeding the access of reacting materials in solution.

The activation energy calculated from the Arrhenius plot (Wang et al. 2011) was 11.48, 10.98 and 7.38 kJ mol^{-1} for the free lipase, P-lipase and C-lipase, respectively. The lower activation energy of the reaction mediated by C-lipase implied that favorable changes in the conformations of the enzyme molecules must have occurred during the covalent coupling.

Conclusions

The Zeta potential test and detection of hydrophobicity showed that PA-M microspheres based on an alkylamine matrix can adsorb lipase through mixed hydrophobic and electrostatic interactions. After optimization of conditions, the maximum loading of lipase was obtained at pH 9.0 and 35°C in 40 min when the initial concentration of lipase was 3.5 mg mL^{-1} in 25 mmol L^{-1} buffer solution. Evaluation of the adsorption process by a Langmuir model and Freundlich isotherm found the adsorption of lipase by PA-M was favorable. Compared to P-lipase, the covalently immobilized lipase (C-lipase) showed better pH stability and thermal stability. C-lipase could retain greater than 85% of its initial activity after 10 rounds of recycling, which was higher than with P-lipase. The catalytic efficiency of lipase reduced after both physical adsorption and covalent immobilization, while the activation energy of lipase was decreased by covalent immobilization, implying favorable changes in the conformations of the enzyme molecules during covalent coupling. PA-M microsphere formed from an alkylamine matrix shows great potential for academic research and industrial applications.

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