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Magnetic nanoparticles supported ionic liquids for lipase immobilization: Enzyme activity in catalyzing esterification

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ABSTRACT

Candida rugosa lipase was immobilized on magnetic nanoparticles supported ionic liquids having different cation chain length (C_1, C_4 and C_8) and anions (Cl^-, BF_4^- and PF_6^-). Magnetic nanoparticles supported ionic liquids were obtained by covalent bonding of ionic liquids–silane on magnetic silica nanoparticles. The particles are superparamagnetic with diameter of about 55 nm. Large amount of lipase (63.89 mg/(100 mg carrier)) was loaded on the support through ionic adsorption. Activity of the immobilized lipase was examined by the catalysis of esterification between oleic acid and butanol. The activity of bound lipase was 118.3% compared to that of the native lipase. Immobilized lipase maintained 60% of its initial activity even when the temperature was up to 80 °C. In addition, immobilized lipase retained 60% of its initial activity after 8 repeated batches reaction, while no activity was detected after 6 cycles for the free enzyme.

1. Introduction

In recent years, nanostructured materials have been used as supports for enzyme immobilization, since the high surface area:volume ratios of nanoparticles can effectively improve the enzyme loading and the catalytic efficiency of the immobilized enzyme [1,2]. However, the recovery of nanoparticle immobilized enzyme is often limited. One of the methods is using magnetic nanoparticles [3–5]. The magnetite-loaded enzymes are easy to recover by a magnetic field, which may optimize operational cost and enhance the product's purity.

Several methods have been reported for the immobilization of lipases on different supports [6] either by covalent binding [7,8], entrapment [9–11], or adsorption [12]. Magnetic microparticles/nanoparticles are also used to immobilize lipase by our group and other groups in the world [13–15]. The properties of supporting materials and the method of immobilization affect activity of immobilized lipase [16–22]. The activity lost of immobilized lipase due to the restriction of conformation change and rigid structure after immobilization remains a great challenge [23].

Ionic liquids are a class of liquids that are composed solely of ions [24]. They are non-volatile, non-flammable, thermally stable, and have excellent salvation properties [25], thus find potential applications in catalysis, electrochemistry, extraction, absorption and so on [26,27]. Recent researches have confirmed that ionic liquids are suitable media for enzyme catalysis [28,29]. Enzymes in ionic liquids exhibit high conversion rates and enantio selectivity. Moreover the stability of enzymes has been proved to be increased over organic solvents. All these advantages in combination with the green, designable properties make ionic liquids a potential material for enzyme immobilization.

In this work, we develop magnetic nanoparticles supported ionic liquids to immobilize lipase with high loading capacity. First, magnetic nanoparticles supported ionic liquids were synthesized by covalent bonding of ionic liquids–silane (Scheme 1) on magnetic silica nanoparticles. Then, *Candida rugosa* lipase was immobilized onto the magnetic supports via physical adsorption. The ionic liquids supports were characterized with scanning electron microscopy (SEM), transmission electron microscopy (TEM), Fourier transformed infrared spectroscopy (FTIR), vibrating sample magnetometer (VSM) and Zeta Potential and Submicron Particle Size Analyzer. The enzyme activity and stability of the immobilized lipase in catalyzing esterification (Scheme 2) were investigated. The functional ionic liquids were not only used for the formation and stabilization of magnetic nanoparticles to immobilize lipase, but also employed as media for lipase catalysis. To our knowledge, there has been no report of synthesis of Fe_3O_4-ionic liquids nanoparticles to apply in enzyme immobilization.

2. Experimental

2.1. General remarks

C. rugosa lipase was purchased from Sigma–Aldrich Company. All other chemicals were purchased from commercial suppliers and

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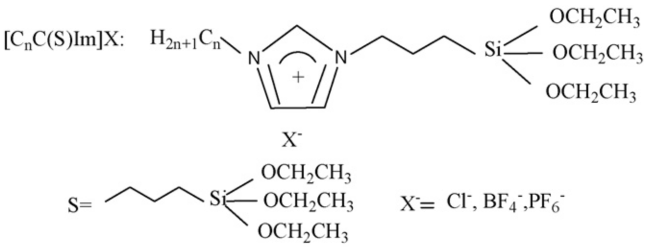
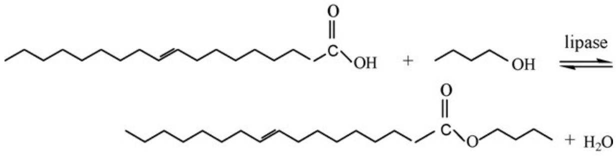
were of the highest purity available. FTIR spectra were recorded on Bruker Vecter 22 FTIR spectrometer, with a deuteriotriglycine sulfate detector and a resolution of 2 cm^{-1}, using KBr pellets. The size and morphology of the particles were observed by transmission electron microscopy (TEM, PHILIPS, FEI TECNAI20) and scanning electron microscopy (SEM, JSM-6700F). A drop of sample was placed on a carbon-coated 200 mesh copper grid, followed by drying the sample at ambient conditions before it was attached to the sample holder on the microscope. The magnetization measurements were performed on a Princeton Applied Research vibrating sample magnetometer model 155 (VSM) at room temperature and the magnetic moment was measured in the magnetic field range of −18,000 Oe to 18,000 Oe. The zeta potentials were measured using Delsa^{TM} Nano Zeta Potential and Submicron Particle Size Analyzer. The pH was adjusted by the addition of HCl or NaOH solution. The results presented are average of five independent measurements. Water content of lipase was determined by Karl Fischer titration (Metrohm, 787, Switzerland, with an accuracy of ±0.0001).



Scheme 1. Functionalized ionic liquids [C_nC(S)Im]X (1-alkyl (methyl, butyl and octyl)-3-(tri-ethoxysilylpropyl)-imidazolium salt, X refers to Cl^-, BF_4^-, PF_6^-).

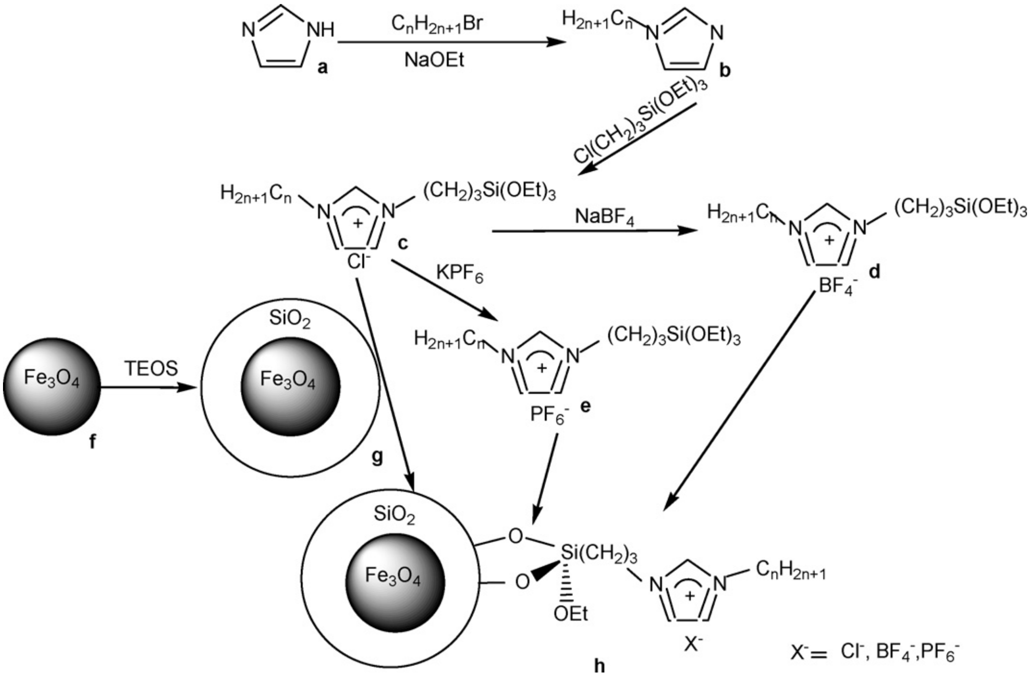


Scheme 2. Ester synthesis between oleic acid and butanol.

2.2. Synthesis of magnetic nanoparticles supported ionic liquids

The magnetite nanoparticles were prepared by the conventional co-precipitation method [30]. 2.33 g $FeCl_3 \cdot 6H_2O$ and 0.86 g $FeCl_2 \cdot 4H_2O$ were dissolved in 100 ml deionized water under nitrogen at 90 °C, then 10 ml 25% $NH_3 \cdot H_2O$ was added with vigorous stirring. After the color of bulk solution turned to black, the magnetite precipitates were separated and washed several times with deionized water and once with 0.02 M sodium chloride by magnetic decantation. Then 2 g water with 40 mg Fe_3O_4 was diluted with 40 ml of water and 160 ml of 2-propanol [31]. This suspension was dispersed under ultrasonification for 10 min. 3 ml ammonium hydroxide solution was added at room temperature in the presence of a constant nitrogen flux, followed by the addition of 0.2 ml tetraethyl orthosilicate (TEOS) with stirring. The mixture was stirred for 12 h to allow the silica shell to grow on the surface of the nanoparticles. The sample was washed with water for several times and dried under vacuum.

Alkyl-substituted imidazole (butyl, octyl) ((b) in Scheme 3) was synthesized according to the procedure described by Pradeep and Frances [32]. Sodium hydride (4.00 g, 100 mmol, 60% suspension in mineral oil) was washed with dry pentane under nitrogen. The reaction flask was placed in an ice bath and distilled THF (30 ml) was added. Imidazole (6.23 g, 91 mmol) was dissolved in 30 ml of THF, and the solution was added into the reaction flask dropwise with stirring under nitrogen atmosphere. The reaction mixture was allowed to react for 2–4 h. Then alkyl bromide (91 mmol) was slowly added at room temperature and the reaction mixture was refluxed for 4–6 h, filtered, washed with fresh THF and filtered again. THF was removed under vacuum, and the liquid residues were distilled under reduced pressure using a 15-cm Vigreux column to give the pure product as a light yellow liquid with an overall yield of 50%. 1-Alkyl-imidazole (50 mmol) and 3-chloropropyl-triethoxy-silane (50 mmol) were stirred at 95 °C for 24 h (yield: 95%). The product (named C_nC(S)Im chloride, where S denotes the silane substitute, and *n* is the number of substitute on 1-C of the imidazolium ring ((c) in Scheme 3)) was washed with ether and dried under vac-



Scheme 3. Synthesis of functional ionic liquids stabilized Fe_3O_4 nanoparticles.

uum at room temperature [33]. The ionic liquid with desired anion (BF_4^- (d), PF_6^- (e) in Scheme 3) was obtained by ion exchange with anion salt according to Mehnert et al. [34]. $C_nC(S)Im$ chloride (50 mmol) was dissolved to 300 ml acetonitrile. Sodium tetrafluoroborate or potassium hexafluorophosphate (50 mmol) was added and the mixture was stirred for 5 days at 30 °C. Then the precipitate was filtrated and the solvent was evaporated in vacuum. Dichloromethane (100 ml) was used to wash the slurry twice and was then evaporated to get the final product with a yield of 80% for BF_4^- and 76% for PF_6^-.

In order to prepare magnetic nanoparticles supported ionic liquids ((h) in Scheme 3), 100 mg magnetic silica was dispersed in toluene by ultrasonication [33,34]. 1 g 1-alkyl-3-(triethoxysilylpropyl)-imidazolium salt was then added to the system and the mixture was stirred at 90 °C for 24 h. After reaction, the solid product was isolated by a magnet, washed with acetonitrile (100 ml) twice and methanol (100 ml) twice, and dried in vacuo.

2.3. Lipase immobilization and desorption

For the immobilization of lipase on magnetic nanoparticles supported ionic liquids, 0–200 mg of lipase was mixed with 100 mg supports in 50 ml phosphate buffer (pH 7.0) [7]. The mixture was shaken at 25 °C for 20–180 min. After that, the solution was removed by a magnet and washed several times with distilled water. When Triton X-100 was used, Triton X-100 in 10 mM phosphate buffer at pH 7 was added with the final concentration of 0.5% [35]. Protein concentration of supernatant before and after the immobilization steps was measured by Bradford's dye binding assay [36]. The amount of lipase was calculated by mass balance. The immobilized lipase was dried under vacuum by Vacuum Freeze Drier. Karl Fischer titration was used to determine the water content of lipase. The water content of immobilized lipase was maintained between 6.9% and 7.4%, similar to that of native enzyme with 7.1%. Desorption measurement was performed by washing the adsorbed lipases using NaCl with different concentration. The desorbed amount of lipase was determined by Bradford's dye binding assay.

2.4. Activity assay of the native and immobilized lipase

The activity of free and immobilized lipase was analyzed by the esterification reactions without solvent. 30 mg lipase (immobilized samples containing the same amount of lipase) was added into 2 ml substrates containing oleic acid and 1-butanol with mole ratio of

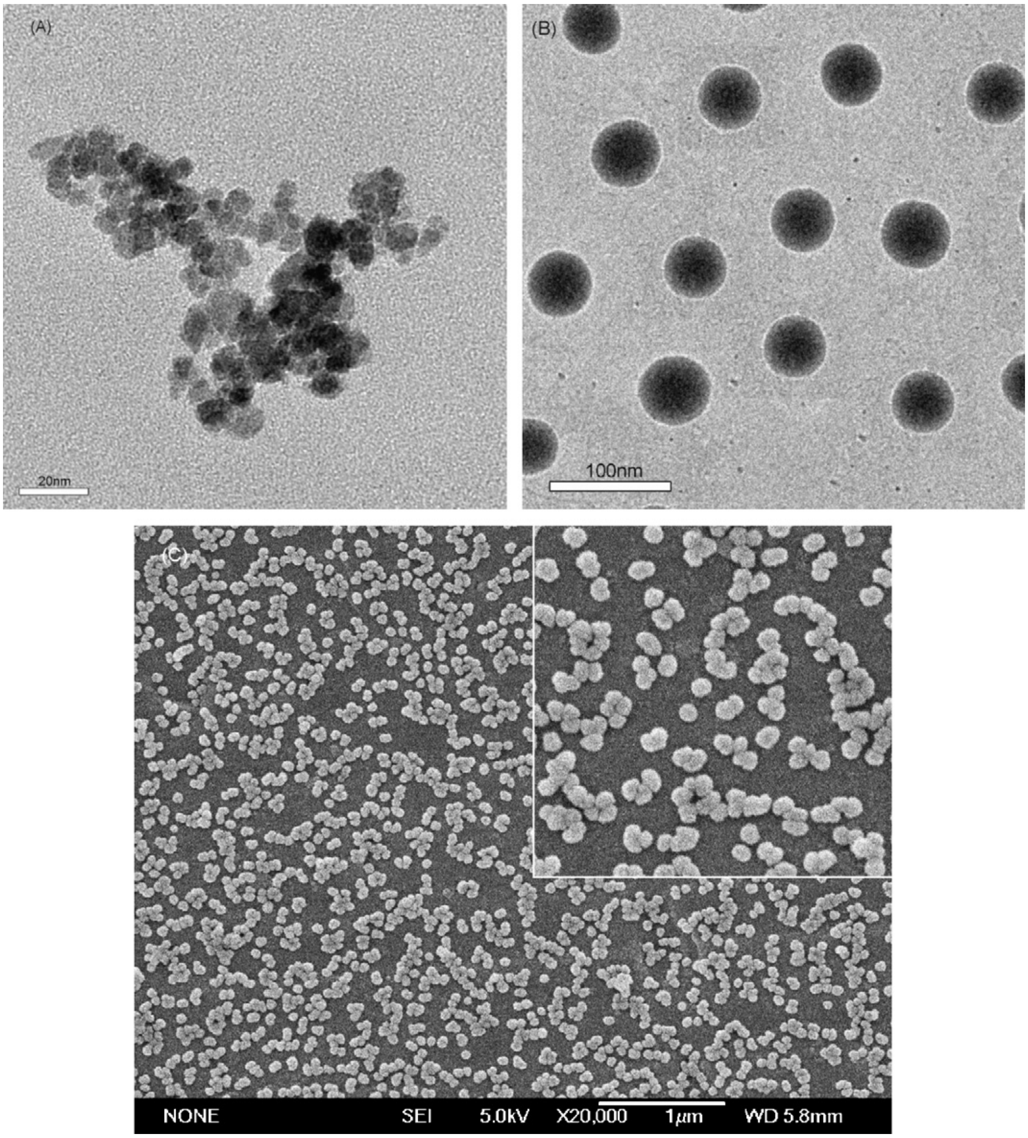


Fig. 1. The micro-pictures of (A) naked Fe_3O_4 particles (TEM); (B) magnetic silica nanoparticles (TEM); (C) magnetic silica nanoparticles modified with ionic liquids $[C_1C(S)Im]Cl$ (SEM).

Table 1
Structures of the substrates 1–5.

Ionic liquids	Molecular formula	X	*n*
1	$[C_1C(S)Im]Cl$	Cl^-	1
2	$[C_1C(S)Im]BF_4$	$BF_4{}^-$	1
3	$[C_1C(S)Im]PF_6$	$PF_6{}^-$	1
4	$[C_4C(S)Im]PF_6$	$PF_6{}^-$	4
5	$[C_8C(S)Im]PF_6$	$PF_6{}^-$	8

1:2. The mixture was reacted in an incubator at 30 °C, 150 rpm for 5 h with 30 min time intervals. The reaction was stopped by the isolation of immobilized lipase using a magnet. The amount of produced ester was measured by titration of the remanent oleic acid using 0.05 M KOH ethanol solution. Each reaction was repeated for three times.

3. Results and discussion

3.1. Synthesis and characterization of functionalized ionic liquids stabilized magnetic nanoparticles

Five different functionalized ionic liquids (ILs 1–5, Table 1) were synthesized and used as coating materials for magnetic silica nanoparticles. The ionic liquids are halogen salts of imidazolium cations substituted at C_1 and C_3 with the desired functional groups. C_1 is alkyl substituted with different carbochain length and C_3 is with triethoxysilane which serves as the bridge between ionic liquids and magnetic particles.

The size and morphology of the resultant nanoparticles were observed by transmission electron microscopy and scanning electron microscopy (Fig. 1). Fig. 1(A) illustrates the TEM picture of Fe_3O_4 particles without modifiers. The average particle size ranged from 8 nm to 10 nm, smaller than the critical size (25 nm) of superparamagnetism. The particles show serious aggregation, while silica coated Fe_3O_4 are well-dispersed (Fig. 1(B)). SiO_2 coating layer could prevent the partial exposure of naked magnetite and thus prevent the aggregation of particles [30]. The SEM pictures of magnetic silica particles stabilized with ionic liquids $[C_1C(S)Im]Cl$ were shown in Fig. 1(C). The particles modified with ionic liquids have a diameter of about 55 nm and show a little aggregation comparing with (B), which might be because the layer of ionic liquids surrounding may interact with each other.

The magnetic properties of these samples characterized by vibrating sample magnetometer were shown in Fig. 2. All of the samples are superparamagnetic, which is important for supporting materials in separation. The saturation magnetization of magnetic silica modified with ionic liquids $[C_1C(S)Im]Cl$, $[C_1C(S)Im]BF_4$, and $[C_1C(S)Im]PF_6$ are 17.3 emu/g, 15.2 emu/g and 11.5 emu/g respectively.

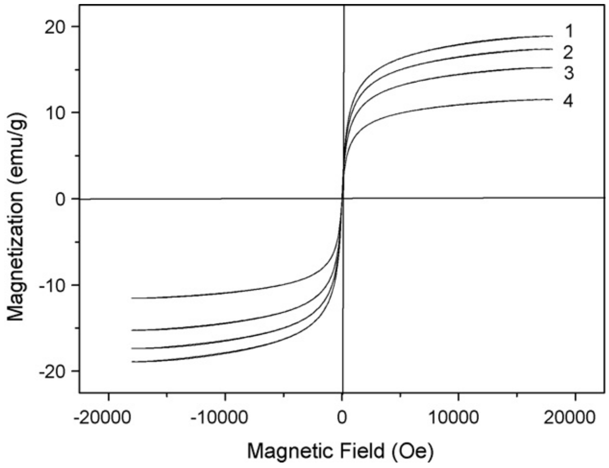


Fig. 2. The VSM of (1) magnetic silica nanoparticles; (2) magnetic silica nanoparticles modified with $[C_1C(S)Im]Cl$; (3) magnetic silica nanoparticles modified with $[C_1C(S)Im]BF_4$; (4) magnetic silica nanoparticles modified with $[C_1C(S)Im]PF_6$.

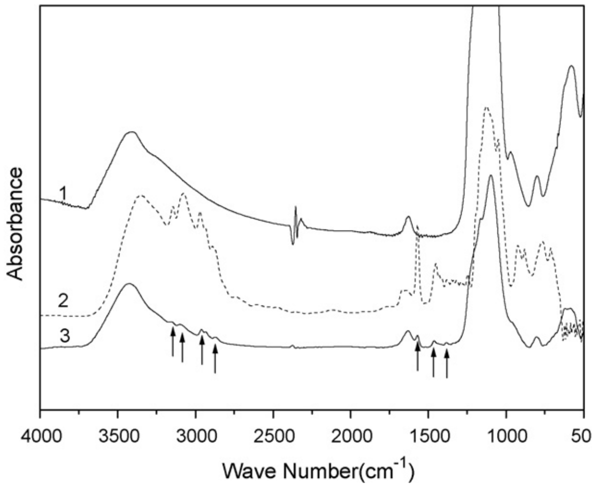


Fig. 3. The FTIR spectrum of (1) magnetic silica nanoparticles; (2) ionic liquids $[C_1C(S)Im]Cl$; (3) magnetic silica nanoparticles modified with $[C_1C(S)Im]Cl$.

Fig. 3 presents the FTIR spectra of magnetic silica nanoparticles (1), ionic liquids $[C_1C(S)Im]Cl$ without (2) and with (3) magnetic silica nanoparticles. For silica coated magnetite, the strong bond at 580 cm^{-1} corresponds to Fe–O vibrations of the magnetite core [37], and the weak bond at 800 cm^{-1} is the characteristic of Si–O–Fe, which implies that SiO_2 is chemically bonded to Fe_3O_4 [38]. Bond at 1100 cm^{-1} represents Si–O bonds, while 3420 cm^{-1}, 1640 cm^{-1} correspond to the stretching and bending vibrations of Si–OH. The IR spectrum of ionic liquids modified magnetic silica shows the characteristic bonds of ionic liquids. 3153 cm^{-1}, 3082 cm^{-1} correspond to C–H stretching vibrations of the imidazolium ring. The relatively weak peaks at 2973 cm^{-1}, 2929 cm^{-1}, 2872 cm^{-1} are the stretching vibrations of CH_3 and CH_2, while 1463 cm^{-1}, 1381 cm^{-1} respond to their bending vibrations. FTIR spectra prove that magnetic silica particles are bonded with ionic liquids.

3.2. Adsorption mechanism

To study the adsorption mechanism, zeta potentials at different pH between supports and lipase were measured, and the results are shown in Fig. 4. The isoelectric point (IEP) of magnetic silica nanoparticles is pH 2.7 similar to previous findings [39]. While for the magnetic silica coated with ionic liquids, IEP increased to pH 9.2. Ionic liquids changed surface electrokinetics of the magnetic supports from anionic to cationic, which is consistent with the findings that imidazolium cation of ionic liquids can form complex with negatively charged surface [40]. The resultant positive surface at neutral pH has strong affinity with negatively charged lipase, whose IEP is 4.5 [41]. The immobilization of lipase on magnetic nanoparticles supported ionic liquids was conducted by ionic adsorption.

The adsorption isotherm is shown in Fig. 5. The amount of loaded lipase increases with increasing the initial concentration of lipase and remains constant at about 63.89 mg/(100 mg carrier). While the enzyme activity reaches its maximum at 119.27 μmol/min/g lipase when the initial lipase concentration is 2.0 mg/ml, and decreases thereafter. This could be due to the steric hindrance of lipase molecules on the surface of the supporting particles, which is severe at high loading density [42]. The amount of lipase immobilized on the particle surface increases with time, and reaches its maximum

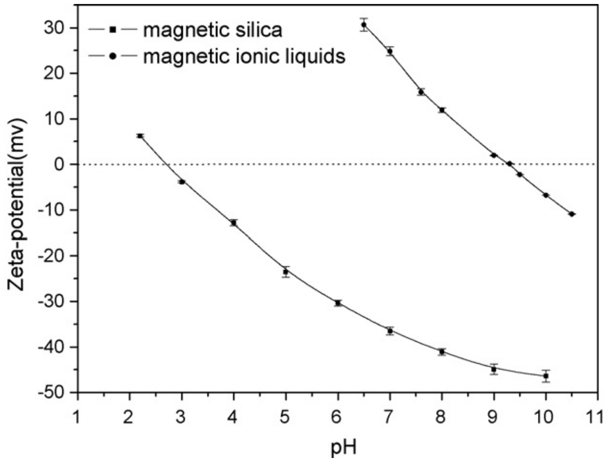


Fig. 4. Zeta potentials of magnetic supports (ionic liquid used was 1-methyl-3-(tri-ethoxysilylpropyl)-imidazolium chloride).

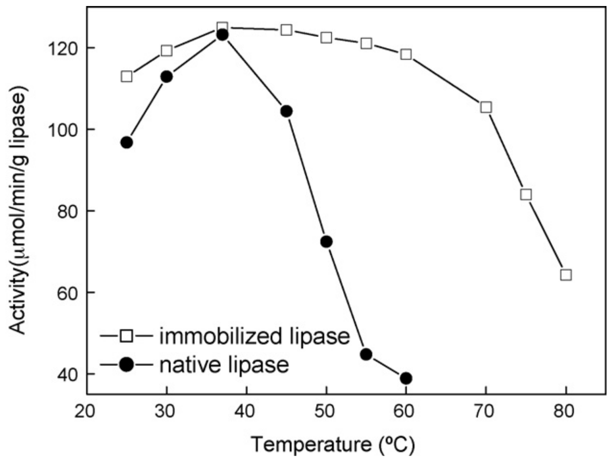


Fig. 6. Effect of temperature on enzyme activity (ionic liquid used was 1-methyl-3-(tri-ethoxysilylpropyl)-imidazolium chloride).

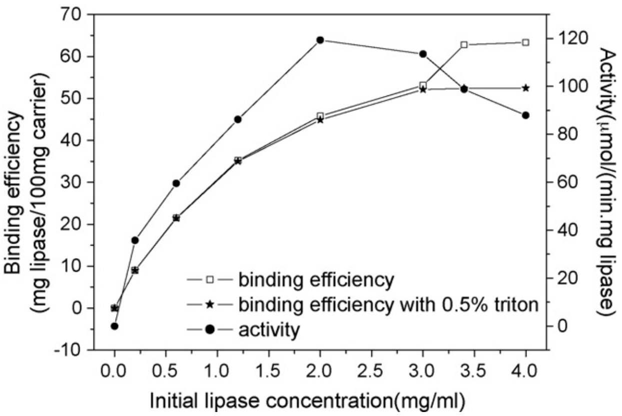


Fig. 5. Effect of initial lipase concentration on loading density and enzyme activity (ionic liquid used was 1-methyl-3-(tri-ethoxysilylpropyl)-imidazolium chloride).

at about 120 min (See supporting information).

The immobilization preparation with 0.5% Triton X-100 is also shown in Fig. 5. It has been reported that lipase immobilized by lipase–lipase interactions can be desorbed from the supports using 0.5% Triton X-100 at a certain concentration [35]. There was no difference in the binding efficiency with or without detergent when the initial lipase concentration was lower than 3.0 mg/ml. However, no more enzymes were loaded with further increasing the initial enzyme concentration. So we assume that lipase–lipase interaction occurred when lipase concentration is above 3.0 mg/ml.

3.3. *Enzyme activity and stability in catalyzing esterification*

Ionic liquids with different cation chain length (C_1, C_4 and C_8) and anions (Cl^-, BF_4^- and PF_6^-) were used to immobilize lipase on magnetic silica particles. Activity of these samples is illustrated in Table 2. All of the immobilized lipase shows higher activity over the native counterpart. Our results also suggest that immobilized lipase activity is affected by cation chain length or anion of ionic Liquids. For the ionic liquids with the same cation $[C_1C(S)Im]^+$, the lipase activities followed the trend $PF_6^- > BF_4^- > Cl^-$. For the ionic liquids with the same anion PF_6^-, the lipase activities increased with increasing the chain length of substitutes on the cation of ionic liquids. As hydrophobicity of anions increased from Cl^-, BF_4^- to PF_6^- [43] while hydrophobicity of cations increased with increasing the chain length of substitutes on the cation, it suggested the immobilized enzyme activity in catalyzing ester synthesis of oleic acid and butanol was related to the hydrophobicity of ionic liquids used. The hydrophobic supports increased the affinity between catalyst and hydrophobic substrates, so the reaction was accelerated [44]. Hence the properties of support can be tuned by changing the structure of cation and anion of the ionic liquids.

Table 2
Lipase activity when immobilized on different ILs modified magnetic nanoparticles.

Catalyzer	Activity (μmol/min/g lipase)	Catalyzer	Activity (μmol/min/g lipase)
Native lipase	106.54	$[C_1C(S)Im]PF_6$	126.87
$[C_1C(S)Im]Cl$	119.27	$[C_4C(S)Im]PF_6$	130.70
$[C_1C(S)Im]BF_4$	125.10	$[C_8C(S)Im]PF_6$	132.33

The effect of temperature (25–80 °C) on the activity of enzyme at pH 7 is shown in Fig. 6. The optimal temperature for both free and immobilized lipase to achieve the highest activity is 37 °C. For free enzyme, activity drops dramatically with increasing temperature, which is only 31.5% of the initial activity at 60 °C. The activity of immobilized lipase is far less influenced by temperature, which remained 60% of its initial activity even when the temperature was up to 80 °C. Enhanced thermal stability of immobilized lipase in this research suggests that the ionic liquids provide good temperature resistance for the enzyme. Rantwijk et al. suggested that ionic liquids could preserve the compact conformation of enzyme, and reduce its rate of destruction of secondary structure at high temper-

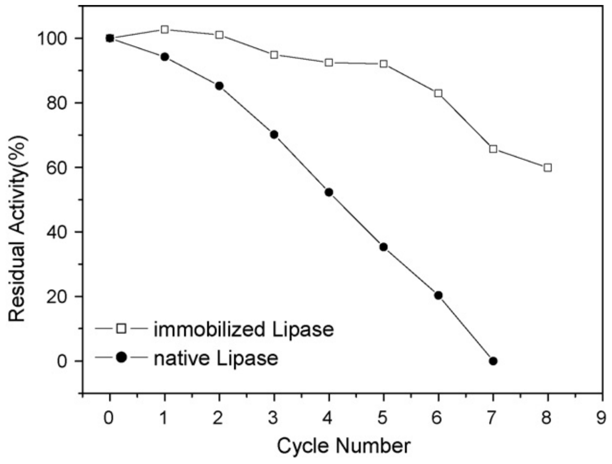


Fig. 7. Reusability of immobilized and native lipase (ionic liquid used was 1-methyl-3-(tri-ethoxysilylpropyl)-imidazolium chloride).

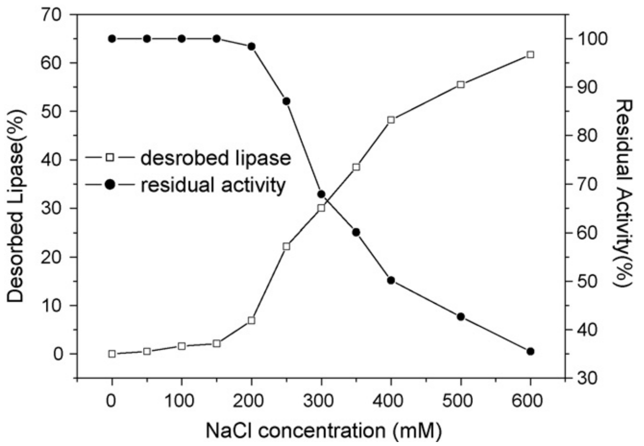


Fig. 8. NaCl desorption effect on the immobilized lipase (ionic liquid used was 1-methyl-3-(tri-ethoxysilylpropyl)-imidazolium chloride).

ature [45]. In addition, the pH behavior of the immobilized lipase has not been changed over its native counterpart (See supporting information).

Another advantage of immobilizing enzyme is the operational stability. As can be seen from Fig. 7, immobilized lipase remained 92% of its initial activity after 5 cycles, while its native counterpart retained only 35%. Such stability might make the use of expensive enzymes more practical.

3.4. Desorption of enzymes

Desorption was conducted under NaCl with different ionic strength. From Fig. 8 we can see that lipase immobilized on magnetic nanoparticles supported ionic liquids can endure 200 mM NaCl and activity remains constant when NaCl concentration was lower than 200 mM. This is much higher than commercial carboxymethyl cellulose as support which lost about 90% of the enzyme when NaCl concentration was only 150 mM [46]. The application of magnetic nanoparticles supported ionic liquids can offer a stable immobilization.

4. Conclusions

In this work, we have synthesized functional ionic liquids modified magnetic nanoparticles to immobilize lipase. Magnetic nanoparticles supported ionic liquids-conjugated enzyme introduced several advantages over the native counterpart. Immobilization saved the amount of both ionic liquids and enzyme used in reactions, facilitating the large scale application. Nanostructured materials can effectively improve the enzyme loading and the catalytic efficiency of the immobilized enzyme because of the high surface area:volume ratios of nanoparticles. Using magnetic nanoparticles could deal with the reuse of immobilized enzyme and ionic liquids, hence the enzyme can be easily recovered and reused for several times without evident loss of activity.

Furthermore, immobilizing lipase with ionic liquids activated the catalytic activity, which was 1.07–1.18 times higher than that for free enzyme. Increasing the carrier hydrophobicity by adjusting the structure of ionic liquids accelerated the enzymatic reaction. Ionic liquids improved the enzymatic resistance against the temperature inactivation. In conclusion, magnetic nanoparticles supported ionic liquids are a kind of novel supports which can activate and stabilize lipase with tunable structure.

Acknowledgments

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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.molcatb.2008.12.001.

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