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Immobilization of Thermostable Lipase QLM on Core-Shell Structured Polydopamine-Coated Fe_3O_4 Nanoparticles

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Abstract: Here, core-shell structured polydopamine-coated Fe_3O_4 nanoparticles were constructed to immobilize thermostable lipase QLM from *Alcaligenes* sp. Systematical characterization indicated that lipase QLM was successfully immobilized on the surface of nanoparticles with an enzyme loading of 21.4 ± 1.47 mg/g immobilized enzyme. Then, the immobilized enzyme was demonstrated to possess favorable catalytic activity and stability in the ester hydrolysis, using *p*-nitrophenyl caprylate as the substrate. Further, it was successfully employed in the kinetic resolution of (*R*, *S*)-2-octanol, and satisfactory enantioselectivity and recyclability could be obtained with an enantiomeric ratio (*E*) of 8–15 over 10 cycle reactions. Thus, core-shell structured polydopamine-coated Fe_3O_4 nanoparticles can be potentially used as a carrier for enzyme immobilization to improve their activity, stability, and reusability, which is beneficial for constructing efficient catalysts for industrial biocatalysis.

Keywords: lipase; immobilization; polydopamine; Fe_3O_4 nanoparticle; core-shell structure; ester hydrolysis; kinetic resolution

1. Introduction

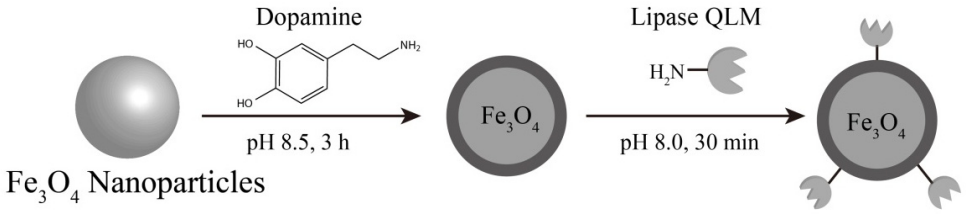
Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) are a group of enzymes capable of catalyzing ester hydrolysis, esterification and transesterification reactions, which makes them promising biocatalysts in the food, pharmaceutical, detergent, and bioenergy industries [1–3]. However, they did not fulfill all the requirements of industrial biocatalysts, such as unsatisfactory stability against thermal, organic solvents, detergents, or metal ions and unfavorable recyclability [4,5]. Enzymes from thermophiles are potential alternatives for solving these obstacles in industrial biocatalysis due to their high catalytic activity and stability under harsh reaction conditions [6]. Among them, thermostable lipase QLM from *Alcaligenes* sp. exhibited high activity of hydrolysis, esterification or transesterification, and favorable stability against thermal and organic solvents [7]. Additionally, it has been successfully employed in the kinetic resolution of racemic compounds, e.g., preparing chiral *sec*-alcohols through enantiomeric transesterification [8,9].

Enzyme immobilization on solid supports is another strategy for improving the stability and recyclability of lipases [10,11]. Simple and efficient carriers play a vital role in constructing effective immobilized enzymes [12]. Iron oxide nanoparticles are one of the most promising platforms for

constructing immobilized enzymes [13–15], owing to their magnetic properties, high specific surface area, easy and low-cost synthesis, and low cytotoxicity. Typical strategies for immobilizing lipase on iron oxide nanoparticles have been dependent on surface grafting through low molecular weight linkers or polymers harboring amino or epoxy groups, which would achieve the immobilization of lipases in a covalent manner [16,17]. However, their applications have been limited by the agglomeration into bigger objects and the sensitivity to acidic and oxidative conditions [18–22]. Additionally, the activity of lipases decreased significantly upon immobilization owing to the influence on enzyme conformation or the access of substrates to the enzyme's active site [23]. The coating of Fe_3O_4 nanoparticles with an outer protective layer is an effective route to avoid these problems. For instance, enveloping them by a polymer or silica to prepare core-shell structured nanocomposites could enlarge the scope of technical applications owing to the unique characteristics of shells including high stability in extreme conditions and introduction of functional groups [24–26]. However, these coating methods for enzyme immobilization such as Fe_3O_4/polystyrene or Fe_3O_4/poly(acrylic acid) were complicated, and it is thus still challenging to explore more facile and green routes for coating Fe_3O_4 nanoparticles and achieving enzyme immobilization.

Dopamine is a well-known biomolecule that can polymerize at an alkaline pH to produce polydopamine [27,28]. The catechol/quinone in polydopamine could facilitate adhesion and deposition on solid surfaces through chelation or hydrogen bonding, which has been used for the coating of polydopamine layers on various colloidal particles [29]. After the coating of a polydopamine layer on solid surfaces, enzyme immobilization or its conjugation with functional ligands possessing nucleophilic groups (e.g., amine and thiol) can be easily achieved through Michael addition and/or Schiff base reactions [30]. Through this method, trypsin, heparin, and albumin have been successfully immobilized on cellulose paper, polyethylene membrane, magnetic nanoparticles, etc. [31–34]. All previous reports have demonstrated that enzyme immobilization through the polydopamine layer was a facial and efficient strategy for obtaining high enzyme loading and favorable activity, stability, and reusability.

In the present study, core-shell structured polydopamine-coated Fe_3O_4 nanoparticles were used as a carrier for immobilizing thermostable lipase QLM, in which the advantages of the intrinsic thermostability of a thermostable enzyme, easy magnetic separation of an iron oxide nanoparticle, and the introduction of function groups through a polydopamine layer were sufficiently combined (Scheme 1). The biocatalytic applications of the immobilized enzyme were then performed to evaluate its activity, stability, and reusability, using the hydrolysis of the *p*-nitrophenyl caprylate (*p*-NPC8) and the kinetic resolution of (*R*, *S*)-2-octanol as models.



Scheme 1. Immobilization of thermostable lipase QLM on core-shell structured polydopamine-coated Fe_3O_4 nanoparticles.

2. Results and Discussion

2.1. Characterization of Immobilized Lipase QLM on Polydopamine-Coated Fe_3O_4 Nanoparticles

As shown in Scheme 1, Fe_3O_4 nanoparticles were first coated with a polydopamine layer via the polymerization of dopamine under alkaline conditions, and the immobilization of thermostable lipase QLM was conducted through the Schiff base reaction between amine groups of lipase and polydopamine as described previously [30]. Arpanaei et al. once constructed a poly(acrylic acid)-coated

Fe_3O_4 cluster@SiO_2 for lipase immobilization, in which complicated procedures were performed through the covalent attachment via *N*-(3-dimethyl aminopropyl)-*N*-ethyl carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) chemistries [21]. Compared with the polymeric layer, the reaction between the polydopamine layer and the lipase was spontaneous and rapid, leading to the enzyme immobilization on Fe_3O_4 nanoparticles soon after mixing. The morphology of the immobilized enzyme was first characterized with transmission electron microscope (TEM). As shown in Figure 1, the polydopamine-coated Fe_3O_4 nanoparticles possessed a spherical structure with a typical core-shell morphology (100–200 nm). After enzyme immobilization, the nanoparticles still exhibited the morphology of a core-shell structure, but a relatively aggregative appearance could be clearly observed. It is still not known how enzyme immobilization affects the aggregation of nanoparticles, and this detailed mechanism will be studied in further research. Afterwards, powder X-ray diffraction (PXRD) was conducted to characterize the immobilized enzyme (Figure 2). All diffraction peaks indicated the presence of magnetite nanoparticles with an inverse spinel phase in the core of the nanoparticles, which is consistent with a previous report [21]. Through the comparison of these PXRD patterns, the crystal structure of the Fe_3O_4 nanoparticles did not suffer any changes upon coating with polydopamine shell and enzyme immobilization. Further, lipase QLM was labeled with fluorescein isothiocyanate (FITC) for preparing FITC-labeled immobilized enzyme, which was observed through confocal laser scanning microscopy (CLSM) in a layer-by-layer scanning mode along the *z*-axis position (Figure 3). Clearly, the fluorescence intensity of FITC showed an increasing tendency from the top to the middle and then decreased from the middle to the bottom. At the top and bottom of the nanoparticles, the FITC-labeled enzyme could be clearly observed to be located on the surface of the nanoparticles, while green fluorescence was detected in a ring manner in the middle of the nanoparticles. These results provide direct evidence for the immobilization of lipase QLM on the surface of core-shell structured polydopamine-coated Fe_3O_4 nanoparticles, owing to the introduction of the functional group of the polydopamine shell.

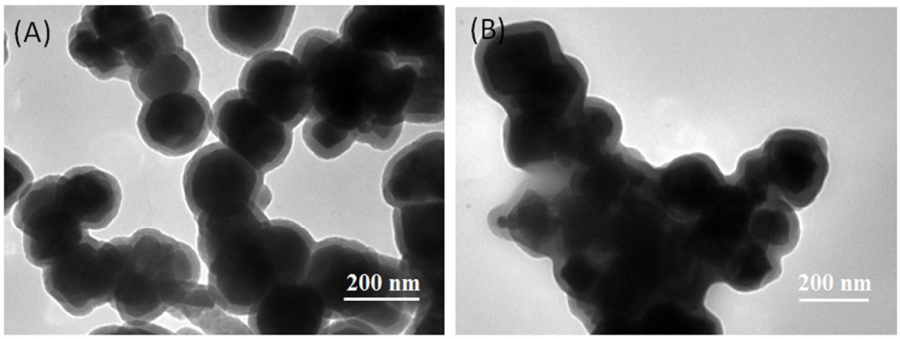


Figure 1. Transmission electron microscopy (TEM) images of polydopamine-coated Fe_3O_4 nanoparticles (**A**) and the immobilized enzyme (**B**).

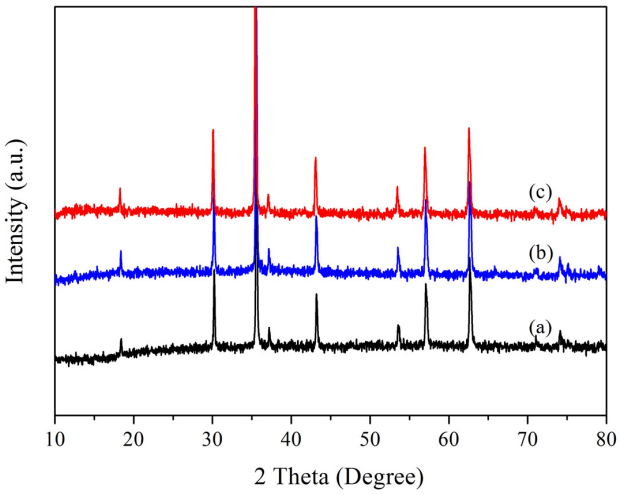


Figure 2. Powder X-ray diffraction (PXRD) patterns of the Fe_3O_4 nanoparticles (**a**); polydopamine-coated Fe_3O_4 nanoparticles (**b**); and the immobilized enzyme (**c**).

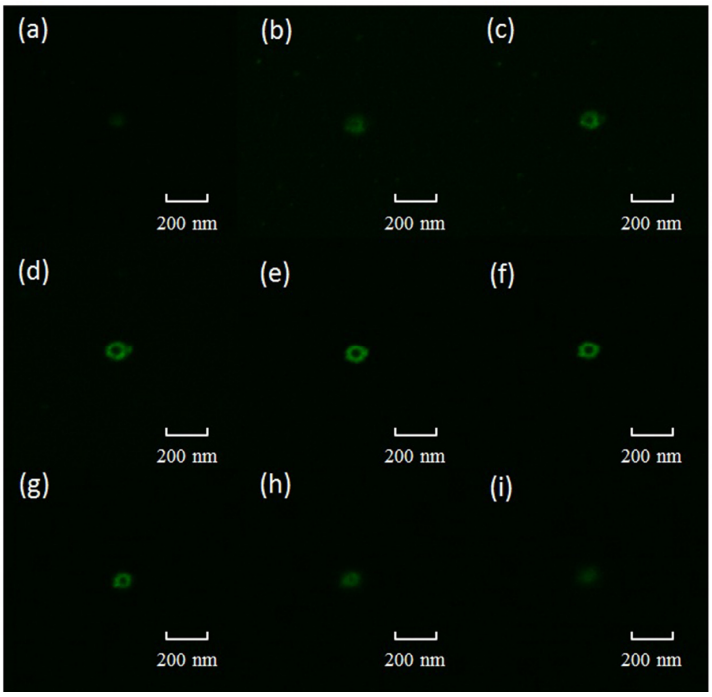


Figure 3. Layer-by-layer confocal laser scanning microscopic (CLSM) photographs of the fluorescein isothiocyanate (FITC)-labeled immobilized enzyme at different scanning depths along the *z*-axis position (**a**–**i**). The fluorescence of FITC was monitored at excitation and emission wavelengths of 488 and 530 nm, respectively.

2.2. Ester Hydrolysis Catalyzed by the Immobilized Enzyme

After the successful construction and characterization of the immobilized enzyme, we first detected the hydrolytic activity using *p*-NPC8 as the substrate. As shown in Figure 4, compared with the control, both Fe_3O_4 nanoparticles and the polydopamine-coated Fe_3O_4 nanoparticles could not achieve the hydrolysis of *p*-NPC8, while the immobilized enzyme could catalyze the hydrolysis of *p*-NPC8 to yield a yellow color as free lipase QLM. Then, the immobilization efficiency as a function of incubation time was monitored through the BCA method (Figure 5). The immobilization efficiency exhibited an obvious increasing tendency with the elongation of reaction time, which reached

57.2% ± 2.33% after 12 h incubation. As the reaction between the polydopamine layer and the lipase was spontaneous and rapid, it has been reported that enzyme immobilization through the strategy of the polydopamine layer could achieve a relatively higher efficiency (73.9%) under optimal conditions for 3 h [34]. Here, the immobilization efficiency was still lower under a similar incubation time (ca. 35.8% at 4 h), which was probably caused by the non-optimal immobilization conditions, especially for a much higher enzyme concentration in the reaction system. After 30 min, enzyme loading was determined to be ca. 21.4 ± 1.47 mg/g immobilized enzyme with an immobilization efficiency of 18.4% ± 1.31%, which has been demonstrated to possess sufficient hydrolytic activity, as shown in Figure 4. Thus, the immobilized enzyme constructed through 30 min incubation was employed as the catalyst for the following research. Subsequently, the effects of the reaction conditions on the enzymatic activity of the lipase QLM and the immobilized enzyme were assayed. As shown in Figure 6A, the lipase QLM and the immobilized enzyme possessed similar temperature profiles, with an optimal temperature of 75 °C. However, different from free lipase QLM (optimal pH of 8.5), the optimal pH value of the immobilized enzyme could not be determined, as it exhibited an increasing tendency with the increasing of pH value (Figure 6B). Additionally, the hydrolysis of *p*-NPC8 was much higher at high alkaline pH, which would influence the accurate measurement of the hydrolytic activity of enzymes. Clearly, Zn^{2+}, Mn^{2+}, Ni^{2+}, and Cu^{2+} showed profound negative effects on the enzymatic activity of free lipase QLM, but the immobilized enzyme possessed favorable tolerance against these metal ions (Figure 6C). As metal ions played key roles in affecting the enzymatic activity, the enhanced activity of the immobilized enzyme in the presence of metal ions would be beneficial for facilitating the application of immobilized enzyme in industrial biocatalysis.

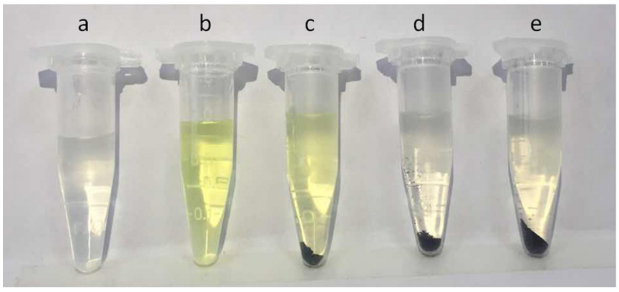


Figure 4. Optical photographs of the hydrolysis of *p*-NPC8 catalyzed by different samples: (**a**) control; (**b**) free lipase QLM; (**c**) immobilized enzyme; (**d**) polydopamine-coated Fe_3O_4 nanoparticles and (**e**) Fe_3O_4 nanoparticles.

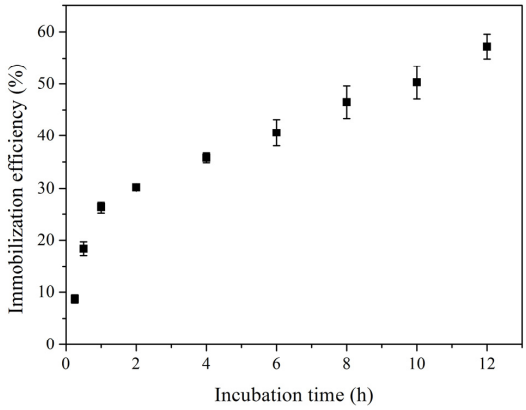


Figure 5. Immobilization efficiency as a function of incubation time. The immobilization was conducted using 1 g of polydopamine-coated Fe_3O_4 nanoparticles and 20 mL of lipase QLM solution (10 mg/mL) at room temperature.

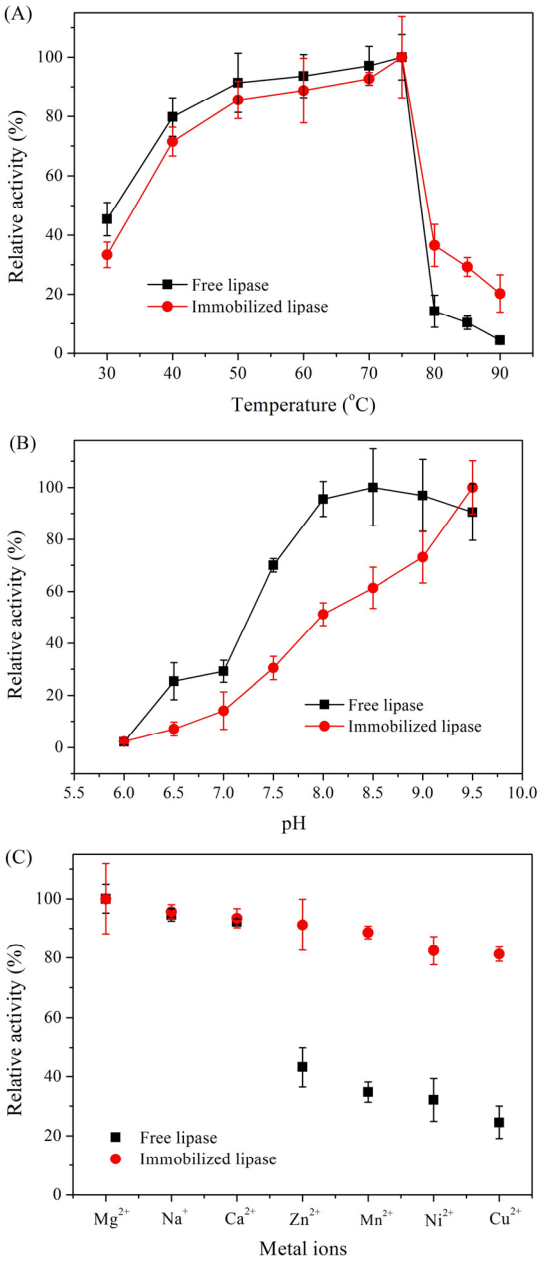


Figure 6. Effects of temperature (**A**), pH (**B**), and metal ions (1 mM) (**C**) on the hydrolytic activity of free lipase QLM (**blank**) and immobilized enzyme (**red**).

Further, the temperature tolerance of the immobilized enzyme was investigated through the pre-incubation of enzymes at 50 °C and 70 °C for different time. As shown in Figure 7A, compared with only 15% of residual activity for free enzyme, the immobilized enzyme could retain ca. 50% of original activity after incubation at 50 °C for 30 min. Notably, a complete loss of activity could be observed for free lipase QLM after incubation at 70 °C for 10 min, while the immobilized enzyme still exhibited a certain activity (ca. 20% of initial activity) (Figure 7B). These results revealed that the immobilized enzyme possessed a relatively stronger tolerance toward temperature and could achieve catalytic reactions at high temperatures, which is favorable for improving its reaction efficiency. In our previous research, we constructed an immobilized lipase QLM in metal–organic frameworks ZIF-8 through biomimetic mineralization with stronger temperature tolerance ability [9], which was probably caused by the embedding of lipase in the porous nanomaterial, not on the surface of immobilization carrier.

The recyclability of the immobilized enzyme was then measured through the hydrolysis of *p*-NPC8 (Figure 8). Clearly, the hydrolytic activity exhibited a declining tendency with the number of cycles, which was probably because the enzyme was immobilized on the surface of the nanoparticles and thus easily dropped from the carrier during the reactions in the aqueous environment. Additionally, enzyme deactivation might also account for the decrease in catalytic activity. However, immobilized enzyme still possessed a certain operational stability in the present research, with ca. 40% of original activity after five cycles. New strategies are still being developed for further improving the recyclability of immobilized enzyme in ester hydrolysis.

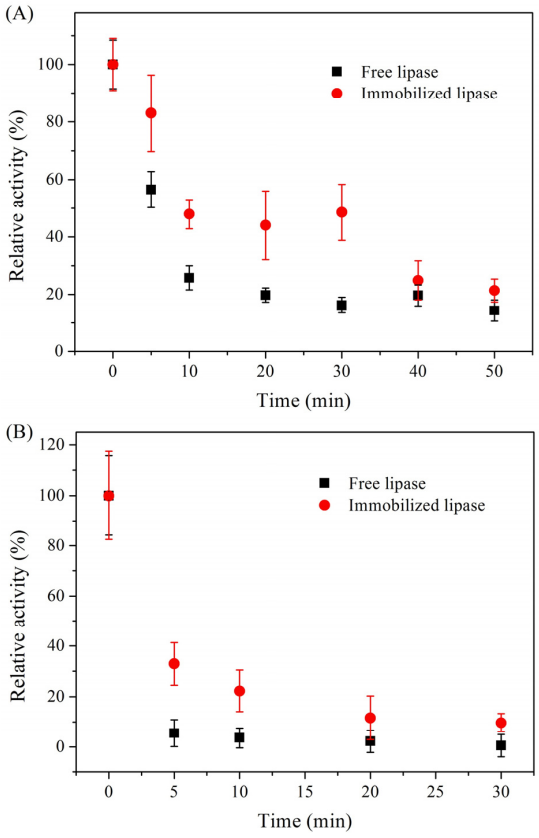


Figure 7. Temperature tolerance of free lipase QLM (**blank**) and immobilized enzyme (**red**) at 50 °C (**A**) and 70 °C (**B**).

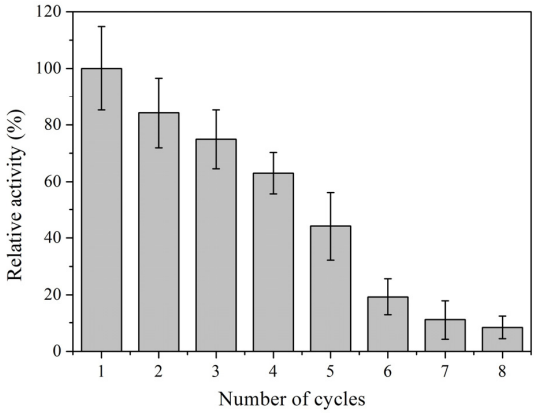


Figure 8. Relative activity of immobilized enzyme in the hydrolysis of *p*-NPC8 for different cycles.

2.3. Kinetic Resolution Catalyzed by the Immobilized Enzyme

Finally, kinetic resolution of (*R*, *S*)-2-octanol was employed as a model to investigate the enantioselectivity and reusability of the immobilized enzyme in a non-aqueous medium. Typical GC chromatogram after enzymatic kinetic resolution was shown in Figure 9, in which peaks at retention times of 45.622, 45.777, 46.844, and 49.734 min represented *R*-2-octanol, *S*-2-octanol, produced *S*-2-octanol ester, and *R*-2-octanol ester, respectively. According to previous reports [9,35], enantiomeric ratio (*E*) could be calculated according to the corresponding peak areas to evaluate the enzyme enantioselectivity. As shown in Figure 10, though the *E* value of the immobilized enzyme showed a slightly declining tendency during enzyme cycles, it was still kept at a stable state with *E* values of 8–15 over 10 cycles. These results elucidated that the immobilized enzyme exhibited favorable enantioselectivity and reusability in the kinetic resolution of *sec*-alcohols, similar to the profile of lipase QLM embedded in metal–organic frameworks ZIF-8 [9]. Different from the catalysis in the aqueous medium, enzyme molecules were not easily desorbed from the immobilization carrier in organic solvents; thus, immobilized enzyme showed a higher recyclability in the kinetic resolution of (*R*, *S*)-2-octanol. This characteristic was potentially beneficial for improving the cost-effectiveness in the enzymatic preparation of chiral compounds.

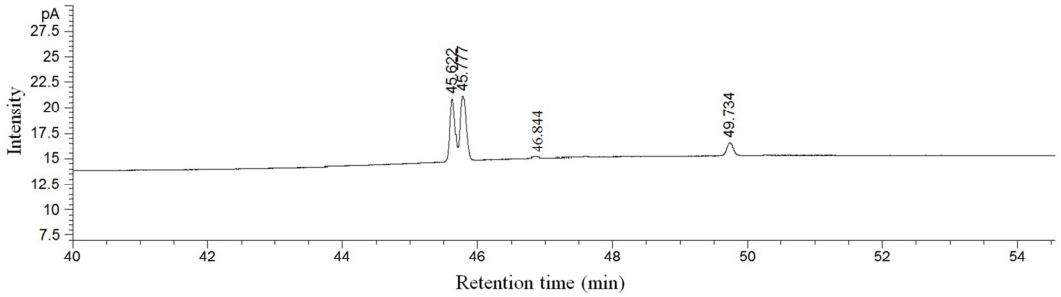


Figure 9. Typical GC chromatogram of the sample after immobilized enzyme-catalyzed kinetic resolution of (*R*, *S*)-2-octanol with vinyl acetate as an acyl donor. The peaks at retention times of 45.622, 45.777, 46.844, and 49.734 min represented *R*-2-octanol, *S*-2-octanol, produced *S*-2-octanol ester, and *R*-2-octanol ester, respectively.

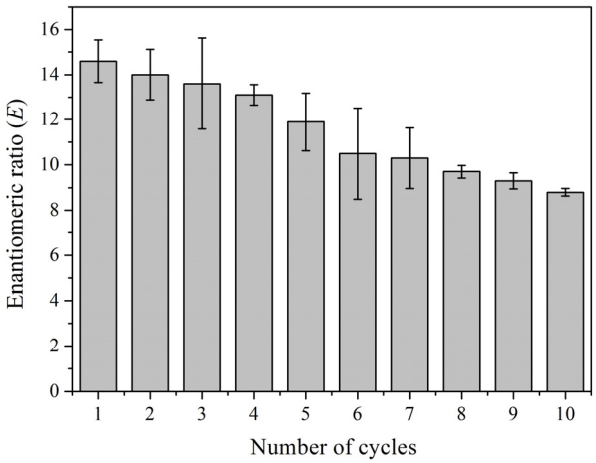


Figure 10. Enantiomeric ratio (*E*) of immobilized enzyme in the kinetic resolution of (*R*, *S*)-2-octanol for different cycles.

3. Materials and Methods

3.1. Materials

Dopamine and Fe_3O_4 nanoparticles were purchased from Aladdin (Shanghai, China) and used as received. Thermostable lipase QLM from *Alcaligenes* sp. was purchased from Meito Sangyo Co., Ltd. (Nagoya, Japan). Fluorescein isothiocyanate (FITC), *p*-NPC8, and (*R*, *S*)-2-octanol were purchased from Sigma-Aldrich (Milwaukee, WI, USA). All other chemicals were of the highest reagent grade commercially available and used as received.

3.2. Immobilization of Lipase QLM on Polydopamine-Coated Fe_3O_4 Nanoparticle

Briefly, 50 mg of dopamine were added to 100 mL of Tris buffer (10 mM, pH 8.5), and 20 mg of Fe_3O_4 nanoparticles were then added to the sample. The mixture was stirred at room temperature for 3 h. The final sample was recovered through magnetic separation and washed with the same buffer twice to obtain polydopamine-coated Fe_3O_4 nanoparticles. The nanoparticles (1 g) were mixed in 20 mL of lipase QLM solution (10 mg/mL in 50 mM phosphate buffer (pH 8.0)). The mixture was stirred at room temperature for different times, and magnetic separation was employed to obtain the immobilized enzyme. Finally, the immobilized enzyme was washed with 50 mM phosphate buffer (pH 8.0) three times and lyophilized for further studies. The immobilization efficiency on the polydopamine-coated Fe_3O_4 nanoparticles was calculated based on the following equation: immobilization efficiency (%) = $[(A - B)/A] \times 100\%$, where A and B represent the protein amount of lipase in the initial solution and the supernatant after magnetic separation, respectively. The protein concentration was measured by a bicinchoninic acid (BCA) method using the Micro-BCA Protein Assay Kit (ComWin Biotech Co., Beijing, China).

3.3. Characterization of Immbozilized Enzyme

The powder X-ray diffraction (PXRD) measurements were carried out by D/MAX2550 diffractometer (Riguku Co., Tokyo, Japan) with Cu Kα radiation (50 kV, 200 mA, λ = 1.5418 Å) and a scanning step of 0.02°. Transmission electron microscopy (TEM) images were acquired using HITACHI-H800 microscope with an accelerating voltage of 200 kV (Hitachi Ltd., Tokyo, Japan).

3.4. Confocal Laser Scanning Microscopic Analysis

The label of lipase QLM using FITC was conducted according to our previous report [9]. Briefly, 150 mg of lipase QLM and 50 mg of FITC were dissolved in 30 mL of phosphate buffer (50 mM, pH 8.0). The solution was stirred at 4 °C for 24 h (180 rpm) and subsequently dialyzed in distilled water for 3 days (molecular weight cut-off: 8000–14000 Da). Lyophilization was then performed to obtain FITC-labeled lipase QLM. The FITC-labeled lipase QLM was immobilized on the polydopamine-coated Fe_3O_4 nanoparticles as described in Section 3.2 to obtain an FITC-labeled immobilized enzyme. The confocal fluorescence images were acquired with confocal laser scanning microscopy (CLSM, Zeiss LSM 710, Jena, Germany) under ambient conditions.

3.5. Ester Hydrolysis Catalyzed by an Immobilized Enzyme

The hydrolytic activity and stability of the immobilized enzyme was investigated using *p*-NPC8 as the substrate [9]. Briefly, 20 μL of *p*-NPC8 solution (10 mM in acetonitrile) was added to 960 μL of phosphate buffer (50 mM, pH 8.0) in a 1 cm path length cell, and the sample was kept at a predetermined temperature (30–90 °C) for 1 min. After the addition of 20 μL of the enzyme solution or the immobilized enzyme, the absorbance changes at 405 nm were monitored with Shimadzu 2500 ultraviolet-visible spectrophotometer equipped with a temperature controller. Using a reference sample of an identical composition except without the addition of enzyme, the background hydrolysis of *p*-NPC8 could be deducted and the amount of enzyme producing

1 μM *p*-nitrophenyl per min was considered as one unit of hydrolytic activity. Similarly, the effects of temperature, pH and metal ions on enzymatic activity and stability were systematically investigated through incubation at different temperatures (30–90 °C), in buffers with different pH values (6.0–9.5) or distilled water containing metal ions (Mg^{2+}, Na^{+}, Ca^{2+}, Zn^{2+}, Mn^{2+}, Ni^{2+}, and Cu^{2+}; 1 mM, chloride salt), respectively. The buffers with different pH values were prepared according to previous reports [9,36]: 40 mM acetic acid, *N*-[2-hydroxyerhyl]piperazine-*N*′-ethane-sulfonic acid (HEPES), *N*-[tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid sodium salt (TAPS), *N*-cyclohexyl-3-aminopropanesulfonic acid (CAPS), and 2-morpholinoethanesulfonic acid (MES). pH values were adjusted with 1 mol/L HCl or 1 mol/L NaOH at 60 °C. The temperature tolerance was assayed through the pre-incubation of enzyme solution at 50 or 70 °C for different times and the subsequent activity determination.

3.6. Kinetic Resolution Catalyzed by the Immobilized Enzyme

The kinetic resolution of (*R*, *S*)-2-octanol with vinyl acetate as an acyl donor was conducted to characterize the catalytic activity and enantioselectivity of immobilized enzyme in non-aqueous medium. Generally, (*R*, *S*)-2-octanol (2 mmol) and vinyl acetate (3 mmol) were added to 1 mL of dichloromethane in a capped reaction vessel. Then, the reaction was initiated by adding 100 mg of the immobilized enzyme. After reacting at 25 °C for 48 h, the immobilized enzyme was recovered by magnetic separation, washed with dichloromethane (2 mL), and used in repeated reuse under the same conditions to evaluate its reusability. Then, the sample was subjected to an Agilent 6890 gas chromatography equipped with an HP-Chiral β column (30 m × 0.25 mm × 0.25 μm, J&W Pharmlab LLC, Levittown, PA, USA) to measure the enantiomeric ratio (*E*) according to previous reports [35]. The injection pool and detector temperature was 300 °C, and the temperature of the column oven was kept at 65 °C for 40 min, and then increased to 95 °C at a rate of 10 °C/min and maintained for 10 min. Hydrogen was used as the carrier gas at an average velocity of 39 cm/s.

4. Conclusions

In this work, we reported a facial method to immobilize thermostable lipase on core-shell structured polydopamine-coated Fe_3O_4 nanoparticles. The immobilized enzyme showed high catalytic activity and stability in the ester hydrolysis and at the same time favorable enantioselectivity and recyclability in the kinetic resolution of *sec*-alcohols, as well as the convenience of being magnetically recovered. These results confirmed that the immobilization of enzymes on magnetic iron oxide nanoparticles by a polydopamine layer is a facial and efficient technique for constructing heterogeneous biocatalysts for potential industrial biocatalysis.

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Author Contributions: Q.L. and Y.W. conceived and designed the experiments; C.W., H.H., W.J., and X.D. performed the experiments; C.W., H.H., W.J., and Y.W. analyzed the data; H.H., W.J., and X.D. contributed reagents/materials/analysis tools; Y.W. and Q.L. wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

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