Superparamagnetic nanoparticle-supported enzymatic resolution of racemic carboxylates†

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Candida rugosa **lipase immobilized on maghemite nanoparticles demonstrated high stereoselectivity in kinetic resolution of racemic carboxylates and improved long-term stability over its parent free enzyme, allowing the supported enzyme to be repeatedly used for a series of chiral resolution reactions.**

Separation of two enantiomers from a racemic mixture is of great importance in many industries, particularly the pharmaceutical industry. This interest is due to the different pharmacokinetic characteristics and pharmacological activities of each enantiomer in a racemic drug.[1] Although numerous approaches have been explored, enzymatic kinetic resolution of racemic mixtures still remains as one of the simplest and most efficient methods to generate enantiomerically enriched stereoisomers in the pharmaceutical and biotechnological industries.[2] Enzymatic resolution reactions employ enzymes as biocatalysts to selectively convert only one enantiomer of a starting material into product. These enzymatic reactions can be carried out under mild conditions and avoid the use of highly toxic heavy metals as catalysts. However, enzymatic resolution reactions have their own limitations. For example, loss of enzymatic activity is frequently observed and some of these biocatalysts are quite expensive for large-scale applications. Immobilization of enzymes onto support matrices has been proven as a successful strategy to overcome many of these problems. The immobilized biocatalysts can be recycled and reused for new reactions, significantly reducing the costs in industrial applications of these enzymes.

During recent years, magnetic nanoparticles have emerged as a new type of matrices for immobilization of enzymes.[3–6] Proteins are usually anchored to a magnetic core such as iron oxide through a linker. Due to the superparamagnetism of the inorganic cores, recovery of these biocatalysts can be facilely achieved by applying an external magnet for magnetic concentration. In addition, nanoparticles have a size in the range of several nanometres, significantly smaller than those of proteins. Unlike larger magnetic micro-beads,[7–11] the small dimensions of nanoparticulate matrices present minimal steric hindrance to reactants in solution for accessing the active sites of biocatalysts, leading to lower mass transfer resistance and less fouling in reactions.

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The use of magnetic nanoparticles for immobilization of enzymes to promote organic transformations has been examined by several research groups recently.[3–6] For instance, magnetic nanoparticle-supported enzymatic esterification and hydrolysis reactions have been investigated.[3–6] However, as far as we know, there has been no report on examining the use of magnetic nanoparticles for supporting enzymatic chiral resolution reactions, which are of particular importance to industrial processes. In this communication, we would like to report our preliminary investigations on magnetic nanoparticle-supported lipase chiral resolution of racemic carboxylates. Our investigations have been designed to find answers to two questions: (1) can high stereoselectivity be achieved in resolution reactions using nanoparticle-supported enzymes? and (2) can nanoparticulate matrices improve the stability of the enzymes, allowing the biocatalysts to be recycled for new rounds of chiral resolution reactions?

The lipase from *Candida rugosa* (E.C.3.1.1.3) was selected for our immobilization and chiral resolution studies. Highly crystalline 11 nm maghemite nanocrystals with narrow size distributions[12,13] were first immobilized with 3-aminopropyltriethoxysilane (ESI†) (Fig. 1). Then, glutaraldehyde was employed as a linker to tether the lipase to the surface of nanoparticles to form Iron Oxide–Lipase. During each steps, magnetic nanoparticles were magnetically concentrated using a permanent magnet, washed with buffer solutions and air-dried. The amount of immobilized enzyme on nanoparticles was obtained by standard BCA protein assays[14] of the original lipase solution, the supernatants, and washing solutions after immobilization, respectively. A loading of 7.5 μg

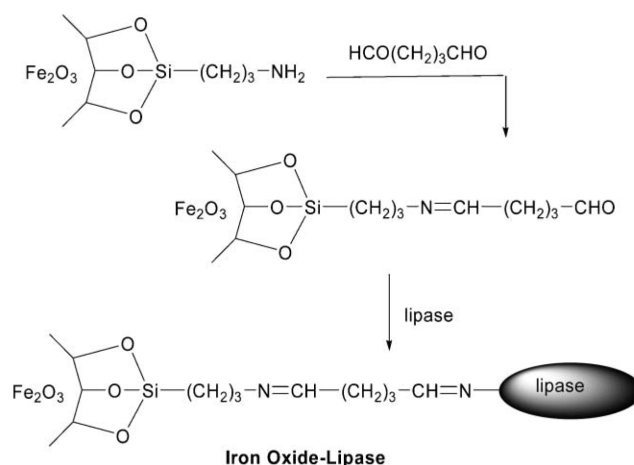

Fig. 1 Schematic representation of immobilization of *Candida rugosa* lipase on maghemite nanoparticles (Iron Oxide–Lipase).

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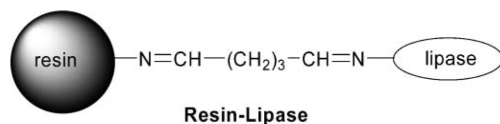

Fig. 2 Structure of *Candida rugosa* lipase immobilized on solid-phase microbeads (Resin–Lipase). Resin–Lipase was used for kinetic resolution of racemic 2-bromopropionic acid (Table 2).

of lipase protein per mg of nanoparticles was obtained through this immobilization strategy.

The enzymatic activity of the supported lipase was determined by closely monitoring the kinetic resolution reactions of three racemic carboxylates (Table 1). At fixed time intervals, small samples were taken out from the reaction mixtures. Iron Oxide–Lipase was magnetically concentrated and removed. The supernatants were then subjected to GC analyses for the conversion progress of carboxylates. The reactions were usually stopped when about 45% of the carboxylates were transformed into esters. The carboxylates and ester products were separated and examined by ^{1}H NMR and Mass Spectrometry analyses. Optical rotation measurements and GC analyses using a chiral GC column were utilized for determining the absolute configurations and enantiomeric excess (ee) values of resolution products. Average isolation yields of 77% and 80% were obtained for *n*-butyl esters and carboxylates, respectively (Table 1). The ee values of isolated carboxylates and esters were usually higher than 99%, comparable to those reported for the non-immobilized, free lipase.[15] Apparently, immobilization of the lipase to nanoparticles did not lead to significant loss of stereoselectivity of the enzyme.

The possibility of recycling the nanoparticle-supported lipase for repeated uses has also been investigated. Racemic 2-bromopropionic acid was chosen as a substrate for four rounds of reactions (Table 2). In a typical experiment, 2-bromopropionic acid was mixed with excessive *n*-butanol in the presence of a catalytic amount of Iron Oxide–Lipase. After seven days, nanoparticles were magnetically concentrated, isolated and washed with buffers. The isolated lipase was then subjected to a new round of the same reaction. Each time, the reaction was stopped after seven days. Supernatants were analyzed by GC to determine the conversion yields of 2-bromopropionic acid in each reaction cycle. Table 2 showed that the conversion percentages dropped from 29% to 20% after four reaction cycles for Iron Oxide–Lipase. In comparison, the free lipase demonstrated much higher enzymatic activity with a

Table 1 Resolution of racemic carboxylates using Iron Oxide–Lipase

R(X)CH–CO_2H —[$CH_3(CH_2)_3OH$, Iron Oxide-Lipase]→ R(H)(X)C–$CO_2(CH_2)_3CH_3$ (I) + R(X)(H)C–CO_2H (II)

			I		II	
Entry	R	X	Yield[a] (%)	Ee[b] (%)	Yield[a] (%)	Ee[b] (%)
1	CH_3	Br	76	99	80	99
2	CH_3	Cl	77	99	78	99
3	$(CH_2)_3CH_3$	Br	80	99	82	92

[a] Determined by GC analyses. [b] Ee determined by chiral GC analyses. Absolute configurations were determined by optical rotation measurements.

Table 2 Recycling of the free lipase and nanoparticle-supported lipase (Iron Oxide–Lipase) for kinetic resolution of racemic 2-bromopropionic acid

Reaction cycle	Conversion[a,d] (%)	Conversion[b,d] (%)	Conversion[c,d] (%)
1	29	55	21
2	21	7	17
3	21	n/a	18
4	20	n/a	17

[a] Catalyzed by Iron Oxide–Lipase. Yields were determined after seven reaction days. [b] Catalyzed by the free lipase. Yields were determined after two reaction days. No products were detected in the third and fourth reaction cycles even after two weeks. [c] Catalyzed by Resin–Lipase (Fig. 2). [d] Conversion yields were determined by GC analyses.

conversion yield of 55% within two reaction days in its first round reaction. However, the catalytic activity of the free enzyme decreased rapidly from 55% to 7% in its second reaction cycle. It completely lost its enzymatic activity in its third and fourth rounds of reaction. On the other hand, the lipase immobilized on micrometre-sized beads (Resin–Lipase) demonstrated long-term stability, but its conversion yields were slightly lower than those of Iron Oxide–Lipase (Table 2).

The enzymatic activity of the nanoparticle-supported lipase in its initial round of reaction is lower than that of the free enzyme. This is probably due to chemical bonding between the proteins and the nanoparticle carriers. The -NH_2 groups that were used for linking to the glutaraldehyde bridge in Iron Oxide–Lipase might play an important role in catalytic reactions. The decrease of enzymatic activity was compensated by the long-term enzyme stability provided by the nanoparticle supports. The nanoparticle-supported lipase could be recovered and reused without significant loss of its activity for 28 days. This is in contrast to the rapid loss of activity of the free enzyme. Our observations support an early report from Ulman on the improved long-term stability of a nanoparticle-supported enzyme.[3]

In summary, maghemite nanoparticle-supported *Candida rugosa* lipase was employed to probe kinetic resolution reactions of racemic mixtures. Chemical immobilization of the lipase to the surfaces of nanoparticles led to a decrease in the enzymatic activity, but this process improved the long-term stability of the enzyme. The nanoparticle-supported lipase could be recycled for subsequent new rounds of reactions, showing no significant drop in its enzymatic activity in repeated reactions. The second advantage of using nanoparticle supports is the facile recovery of the immobilized enzyme. An external small permanent magnet that is inexpensive and readily available from many commercial sources can be utilized for magnetic concentration of the enzyme in high efficiency. More importantly, our work here, for the first time, demonstrated that the nanoparticle-supported lipase maintained high stereoselectivity towards chiral resolution reactions. The ee values of the nanoparticle-supported resolution products were comparable to those of the free enzyme. This high stereoselectivity in conjugation with the long-term stability and facile recovery of magnetic nanocluster-supported enzymes will make economically viable the use of magnetic nanoparticles for the industrial chiral resolution processes. Such magnetic nanometre-sized carriers can also be potentially utilized for supporting other types of large-scale organic and biological transformations in the pharmaceutical and

biotechnological industries. More detailed investigations in this area, for example, studies of nanoparticle structure and substrate effects on the catalytic activity of the immobilized biocatalysts, are in progress and will be reported in due course.

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