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Facile preparation of recyclable biocatalyst-decorated magnetic nanobeads in aqueous media

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ABSTRACT

A facile process was developed to manufacture biocatalyst-conjugated magnetic nanobeads, which afford no loss of the intrinsic activity and enantioselectivity of biocatalysts. Up to 90% of their activities remained after six-time recycling in aqueous media.

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Immobilization is a conventional approach for improving stability and recyclability of enzymes.[1] Most techniques for immobilization of enzymes are based on physical adsorption on porous supporting materials, which contain a large surface area. Adsorption of enzymes on such materials can decrease a tendency for aggregation of enzymes and thereby sustaining the active forms in organic solvents. However, enzymes immobilized by this method are not recyclable when the reaction is performed in aqueous media on account of enzymes' leaching out of the supporting matrix. Covalent immobilization can be one of the alternative approaches for recycling biocatalysts in aqueous media. Nevertheless, covalent linkage of enzymes on the macro-sized porous polymer materials causes mass transport limitation because the interior enzymes in the porous materials may have less or slow contact with substrates. Mass transport limitation results in retardation of reactions as well as decrease of enantioselectivity.[2] Instead, utilizing nano-sized polymer particles as a supporting material can reduce mass transport limitation, but it is often problematic to recover the particles by centrifugation from aqueous media due to their low density.[3] To overcome these drawbacks, using nano-sized magnetic particles as a supporting material has been proposed.

Protein-decorated magnetic nanoparticles have garnered more attention in biotechnology, such as isolation of proteins, enhancement of magnetic resonance imaging, and immobilization of enzymes.[4] Nano-sized magnetic particles possess a greatly enhanced surface area, which affords better contact with substrates without mass transport limitation. In addition, one of the most distinctive characteristics of magnetic nanoparticles over polymer nanobeads in bio-applications is that they are conveniently separable from reaction media by applying an external magnetic field or by simple centrifugation. Although several enzymes, such as hydrolase,[5] glucose oxidase,[6] alcohol dehydrogenase,[7] and chloroperoxidase,[8] have been directly or indirectly immobilized on magnetic nanoparticles, many cases do not provide sufficient activity or require a complicated process for manufacturing. For example, direct conjugation of a lipase (*Candida rugosa* lipase) to magnetic nanoparticles showed 236-fold activity decrease compared to the free form of the lipase.[5a] Silica- or polymer-entrapped enzymes on magnetic particles showed improved activity and stability, but additional processes after encapsulating are required to functionalize the surface of the silica or polymer shell for protein conjugation.[5c,8] Herein, we describe a facile process of covalent immobilization of biocatalysts on polymer-encapsulated magnetic nanoparticles with maintaining comparable activity to the free enzymes in aqueous media.

The process of manufacturing enzyme-conjugated magnetic nanobeads is straightforward and simple compared to the previously reported methods[5–8] (Fig. 1). The current approach introduces a functional group to be used for enzyme conjugation during the encapsulation step of polymethylmethacrylate (PMMA)

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on magnetic nanoparticles, whereas the previous methods required additional processes to introduce such functional groups.

The magnetic nanoparticles (Fe_3O_4) were prepared by coprecipitation of Fe(II) and Fe(III) ions in a basic solution. The precipitated magnetic particles were then coated with oleic acid to enhance

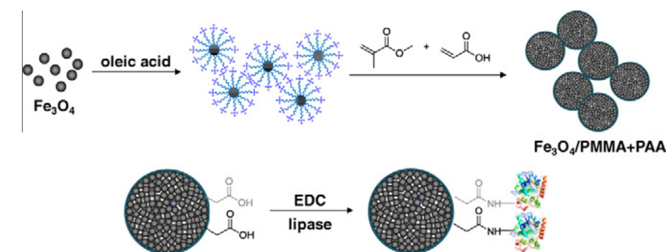

Figure 1. Process for preparation of lipase-conjugated magnetic nanobeads.

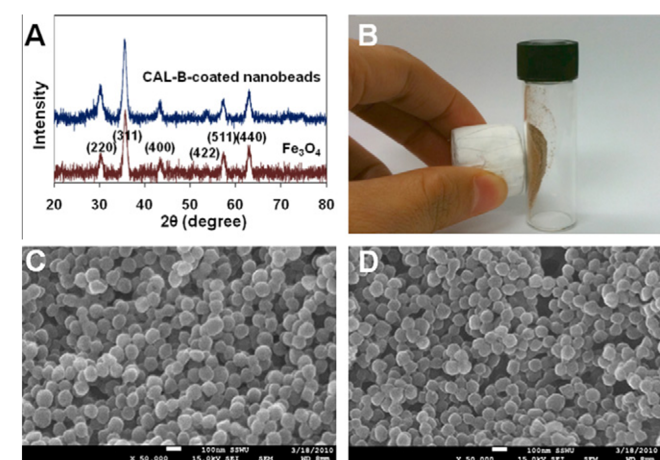

Figure 2. (A) PXRD patterns of Fe_3O_4 and PMMA-encapsulated magnetic beads, (B) picture showing magnetic attraction, (C) FE-SEM image of the polymer-coated magnetic nanobeads, (D) FE-SEM image of the CAL-B-conjugated magnetic nanobeads. Scale bar = 100 nm.

interaction with polymers. Initially, we encapsulated the magnetic particles with polystyrene (PS) containing carboxylic-acid groups by emulsion polymerization in an aqueous solution. The PS-coated magnetic beads were uniform, but an average diameter was submicron-sized (~300 nm, Fig. S1 in the Supplementary data). Presumably, the phenyl groups in PS are strongly amassed in water and thereby the particle sizes become larger. In order to reduce the size of beads and thereby to provide a larger surface area to volume ratio, we used methyl methacrylate, which has lower aggregation propensity in water. The encapsulation process of PMMA was achieved by radical-initiated polymerization using methyl methacrylate and acrylic acid in an aqueous methanolic solution (see the Supplementary data). Powder X-ray diffraction analyses indicate that the polymer encapsulation does not affect the structural integrity of Fe_3O_4 (Fig. 2A), and the nanobeads can be attracted by applying an external magnetic field and thus separable from the reaction media (Fig. 2B). Field-emission scanning electron microscopy (FE-SEM) analyses of the resulting magnetic nanobeads revealed that the magnetic nanobeads are uniformly spherical and an average diameter of the PMMA-coated beads is estimated to be ~81 nm (Fig. 2C and Fig. S2a in the Supplementary data).

Commercial seven lipases and one protease (see the list and their abbreviations in the Table 1) were then conjugated to the PMMA-encapsulated magnetic nanobeads after activation of the carboxylic-acid groups on the beads by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). The EDC activation of the carboxylic-acid groups on the surface of the PMMA-encapsulated magnetic nanobeads allows formation of an amide bond with the free amino groups (i.e., the N-terminal or the side chain of lysine) of enzymes. The morphology of the lipase-conjugated magnetic nanobeads was not altered but the diameter became slightly larger (~84 nm, CAL-B-conjugated beads shown as a representative in Fig. 2D and Fig. S2b in the Supplementary data). The protein amount in a solution was measured by the Bradford protein assay[9] and the amount of immobilized enzyme was determined to be 0.75–0.91 mg g^{-1} by difference in the protein amount of the supernatants before and after incubation (Table S1 in the Supplementary data).

Table 1
Hydrolysis of lipase-conjugated magnetic nanobeads toward *rac*-1-phenylethyl butanoate[a]

rac-phenylethyl butanoate → (Enzyme-conjugated nanobeads (30mg), pH7.4 buffer, 30 °C, 24h) → (*R*)-1-phenylethyl alcohol + (*S*)-1-phenylethyl butanoate + HO(C=O)*n*-Pr

Entry	Enzyme	ee_s (%)	ee_p (%)	Conversion[b] (%)	*E*[c]
1	Lipase B from *Candida antarctica* (CAL-B) (20 mg)	>99.9	>99.9	50	>200
2	Lipase B from *Candida antarctica*, free form	97.4	>99.9	49	>200
3	Lipase from *Burkholderia cepacia* (BCL)	>99.9	>99.9	50	>200
4	Lipase from *Burkholderia cepacia*, free form	53.5	>99.9	35	>200
5	Lipase from *Candida rugosa* (CRL)	80.9	69.8	54	13.7
6	Lipase from *Candida rugosa*, free form	59.8	53.2	53	5.9
7	Lipase type I from Wheat Germ (WGL)	3.8	1.3	75	1.1
8	Lipase from *Rhizopus arrhizus* (RAL)	9.5	89.7	10	20.2
9	Lipase from *Mucor javanicus* (MJL)	14.5	80.7	15	10.8
10	Lipase from *Thermomyces lanuginosus* (TLL)	33.1	98.3	23	156.8
11	Protease S	<0.5	<0.5	<1	n.d.[d]

[a] Condition: *rac*-1-phenylethyl butanoate (0.05 mmol) and enzyme-conjugated nanobeads (30 mg) except CAL-B (20 mg) or the corresponding amount of proteins of free enzymes (17–27 μg) was dissolved in a buffer (1× PBS, 1 mL, pH 7.4), and the reaction mixture was shaken at 200 rpm and 30 °C for 24 h.
[b] The values of conversion were calculated using the measured enantiomeric excess of the starting material (ee_s) and product (ee_p).
[c] *E* = enantiomeric ratio as defined by Chen, C. S.; Fujimoto, Y.; Girdaukas, G.; Sih, C. J. *J. Am. Chem. Soc.* **1982**, *104*, 7294–7299. For high enantioselective reactions (i.e., $E \geqslant 200$), the maximum conversion is 50%.
[d] n.d. = not determined.

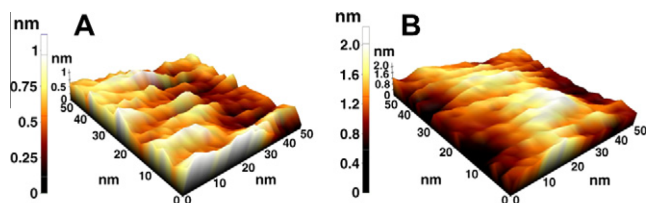

Figure 3. The AFM images of a magnetic nanobead: (A) A PMMA-coated magnetic nanobead, (B) a CAL-B-conjugated nanobead.

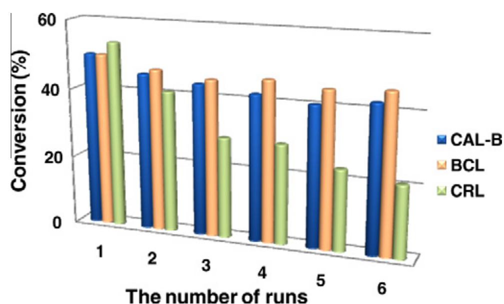

Figure 4. Recycling of the lipase-conjugated nanobeads in aqueous media. The lipase-conjugated beads were recovered by a magnet for consecutive runs.

In order to investigate the detailed morphologies of the surfaces of a single magnetic nanobead, three dimensional atomic force microscopy (AFM) images were obtained (Fig. 3). The surface of a lipase-conjugated nanobead contains fewer valleys and the valleys have longer peak-to-valley distance compared to that of a PMMA-encapsulated magnetic bead, although the individual lipase molecules could not be detected.

For evaluation of the catalytic efficiency of the immobilized lipases on the magnetic nanobeads, we measured the reaction conversion as well as enantioselectivity toward hydrolysis of *rac*-1-phenylethyl butanoate in an aqueous solution as a model reaction (Table 1). The kinetic resolution of *rac*-1-phenylethyl alcohol by lipases has been extensively studied and well documented in the literature.[10] The immobilized lipases catalyzed the hydrolysis with 10–75% conversion (entries 1–10) but protease S showed no conversion (entry 11). Protease S does not accept *rac*-1-phenylethyl butanoate as a substrate rather than becoming inactive by immobilization because even the hydrolysis catalyzed by the free form of protease S did not occur (data not shown). In addition, the reaction of protease-S-conjugated nanobeads with *p*-nitrophenylacetate as a substrate showed equivalent activity to that of the free enzyme (Fig. S3 in the Supplementary data). Among seven lipase-conjugated nanobeads, CAL-B-, BCL-, and CRL-conjugated ones showed high conversion as well as high enantioselectivity (entries 1, 3, and 5, respectively). The reactions catalyzed by the free form of those three lipases were also carried out to check the changes in the enzyme activities caused by the current immobilization method (entries 2, 4, and 6). Interestingly, the reactions by the lipase-conjugated nanobeads in this study showed comparable conversions to the reactions by the free lipases, although it is generally reported that the activity of covalently immobilized enzyme decreases.[5,11]

In addition, we conducted recycling experiments of the three lipase-conjugated magnetic nanobeads. The lipase-conjugated beads were isolated by a magnet for consecutive runs after a reaction had been completed. As shown in the Figure 4, the conversion in the reactions by CAL-B- and BCL-conjugated magnetic nanobeads was consistent during six-time recycling with maintaining the same enantioselectivity ($E \geqslant 200$). For the CRL-conjugated magnetic nanobeads, it was observed that the conversion decreased by 5% at each run, but the value is still smaller than the activity loss (~15% at each run) in the previous report,[5a] although Liu and coworkers reported lower losses (~10% loss after six runs) in recycling experiments of hydrolysis at the oil/water interface by *Candida cylindracea* lipase immobilized on oleic-acid–Pluronic-coated magnetic particles.[8a]

In conclusion, our approach demonstrates that the introduction of hydrophilic polymers provides advantages in retaining the intrinsic activity of enzymes over direct coating of enzymes to the magnetic nanoparticle surface in covalent immobilization of enzymes. This study also showed that introducing a functional group to be used for enzyme conjugation during the polymer coating step reduces the effort for overall manufacturing processes.

Acknowledgments

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Supplementary data

Supplementary data (experimental details, analysis of the PS-coated magnetic beads by SEM, measuring diameters of the magnetic beads and hydrolysis of *p*-nitrophenyacetate by immobilized protease S) associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2010.12.099.

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