
**Journal of Macromolecular Science, Part A**
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597274

# Resolution of (*D, L*)-Phenylalanine in Biphasic System by α-Chymotrypsin Immobilized on Superparamagnetic Nanogels

Jiahui Yu[a]; Chengyun Wang[a]; Jun Hong[a]; Jin Huang[ab]
[a] College of New Drug Innovation Research & Development, East China Normal University, Shanghai, P. R. China [b] College of Chemical Engineering, Wuhan University of Technology, Wuhan, P. R. China

**To cite this Article** Yu, Jiahui , Wang, Chengyun , Hong, Jun and Huang, Jin(2009) 'Resolution of (*D, L*)-Phenylalanine in Biphasic System by α-Chymotrypsin Immobilized on Superparamagnetic Nanogels', Journal of Macromolecular Science, Part A, 46: 10, 943 — 948
**To link to this Article: DOI:** 10.1080/10601320903158263
**URL:** http://dx.doi.org/10.1080/10601320903158263

*Journal of Macromolecular Science®, Part A: Pure and Applied Chemistry* (2009) **46**, 943–948

ISSN: 1060-1325 print / 1520-5738 online
DOI: 10.1080/10601320903158263

Taylor & Francis
Taylor & Francis Group

# Resolution of (*D, L*)-Phenylalanine in Biphasic System by α-Chymotrypsin Immobilized on Superparamagnetic Nanogels

JIAHUI YU[1], CHENGYUN WANG[1], JUN HONG[1] and JIN HUANG[1,2,*]

[1]*College of New Drug Innovation Research & Development, East China Normal University, Shanghai 200062, P. R. China*
[2]*College of Chemical Engineering, Wuhan University of Technology, Wuhan 430070, P. R. China*

Received March 2009, Accepted April 2009



α-Chymotrypsin immobilized on super paramagnetic nanogels covered with carboxyl groups, exhibited high stereoselectivity in kinetic resolution of racemic phenylalanine in toluene/water biphasic system, and even improved reusability over its parent free enzyme. The results also showed that the addition of ionic liquid to water phase could enhance the stereoselectivity of the immobilized enzyme to a certain extent while the conversion yield decreased. The long-term stability allows the supported enzyme with nano-scaled dimension to use in continuous chiral resolution reaction, which opens a new horizon for enzymatic chiral resolution in large-scale production.

**Keywords:** Superparamagnetic nanogels, enzymic resolution, immobilized α -chymotrypsinc, (*D*, *L*)-Phenylalanine, ionic liquid

## 1 Introduction

Resolution of two enantiomers from a racemic mixture is of great importance in pharmaceutical industry. Nowadays, a plethora of interest on this subject arises from the fact that isomer bears different pharmacokinetic characteristics and pharmacological effects (1). One enantiomer usually provides the desired activity, while the opposite enantiomer may be inactive or sometimes toxic. Except for the separation methods, enzymatic kinetic resolution has been proven to be the simplest and most efficient method to achieve chiral resolution of a racemic mixture (2–4). Commonly, enzyme-catalyzed reactions have higher selectivity, in comparison to the reactions driven by chemical catalyst, produce a cleaner product and achieve higher yield. Additionally, these reactions can be carried out under mild conditions. However, the enzymatic resolution reactions still suffer from numerous limitations. For instance, loss of activity is frequently observed since enzymes are labile molecules. On the other hand, free enzymes are difficult to be retrieved from reaction media. This could greatly increase the operational costs, especially for expensive enzymes in large-scale applications. As a result, recycling of immobilized enzymes becomes the central issue of enzymatic catalysis. Indeed, immobilization of enzymes onto supports has been corroborated to be a robust strategy to overcome the drawbacks aforementioned. Immobilization provides a preferable approach to make enzyme reuse and to extend its shelf half-life.

Currently, a large number of nano-scaled carriers have been applied in enzyme immobilization (5–10). Among the carriers, functional superparamagnetic nanoparticles have become increasingly attractive due to their superparamagnetic feature (11–14). In general, proteins are anchored to the magnetic nanoparticles through functional groups on the surface of the magnetic nanoparticles (15–17).

Magnetic nanogels, a class of inorganic/polymer core-shell composites with nano-scaled dimension, comprise a core of magnetically susceptive nanoparticle and a shell of crosslinked polymeric network. They usually possess an excellent swollen capacity, functional groups and hydrophilicity. The use of superparamagnetic nanogels as supports for enzyme immobilization is endowed with the following advantages: (i) higher specific surface favoring the binding efficiency, (ii) lower mass transfer resistance and less fouling, (iii) readily separation and lower operation cost. Consequently, magnetic nanogels would be an optional support for enzyme immobilization.

Magnetic immobilized enzymes have been extensively examined recently. Especially, magnetic nanoparticle-supported enzymatic esterification and hydrolysis reactions

*Address correspondence to: Jin Huang, College of New Drug Innovation Research & Development, East China Normal University, Shanghai 200062, P. R. China and College of Chemical Engineering, Wuhan University of Technology, Wuhan 430070, P. R. China. E-mail: huangjin@iccas.ac.cn

have been explored in organic solvents (18–21). However, to our knowledge, there is little literature reported on nanoparticle-supported enzymatic resolution in biphasic system. Herein, we would like to report our preliminary investigations on the lipase-catalyzed kinetic resolution of racemic phenylalanine in toluene/water biphasic system. It was anticipated that this work could open a new gateway for production of optically pure amino acid in large scale.

## 2 Experimental

### 2.1 Materials

Methylacrylic acid (MAA), *N*,*N*′-Methylene-bis-(acrylamide) (MBA), *N*-hydroxysuccinimide (NHS, Su-OH) and 1-ethyl-3-(3-dimethylaminepropyl) carbodiimide hydrochloride (EDC· HCl), ethanol amine and Ionic liquid ($[Me_3NC_2H_4OH]^+$ $[ZnCl_3]^-$) were purchased from Shanghai Chemical Reagents Corp.. *N*-Benzoyl-*L*-Tyrosine Ethyl Ester (BTEE) and DL-phenylalanine methyl ester were purchased from Sigma. BCA protein assay kit and $\alpha$ -Chymotrypsin (CT) were obtained from Biotian Company Ltd., China. $Fe_3O_4$ nanoparticles were prepared by a partial reduction method according to references (22, 23). Their sizes were about 10 nm in diameter with a polydispersity of 0.061 measured by dynamic light scattering (DLS) (Zetasizer Nano ZS, Malvern Instruments, UK). Saturation magnetization was measured to be 66.3 emu · $g^{-1}$. Immeasurable coercivity and remanence showed its super-paramagnetic profile. MAA and MBA were purified prior to use. Other chemicals were all of analytic grade and used without further purification.

### 2.2 Synthesis of Carboxyl-Functionalized Magnetic Nanogels

The carboxyl-functionalized magnetic nanogels were synthesized according to our previous methods (24). Briefly, MAA (1 mL, 11.8 mmol and 1% MBA (2 mL, 64.9 $\mu$mol) were mixed in 120 mL water, and adjusted pH to 6 with 5 M NaOH before charging into the quartz flask. The reaction system was bubbled with nitrogen for 10 min. Subsequently, 2 mL of magnetofluid (10 mg $Fe_3O_4$ per mL) was dropped into the flask. The mixture was irradiated with 500 W of xenon lamp for 1 h under nitrogen atmosphere. After, the resultant magnetic nanogels were recovered by a 0.5 T of magnet, and washed several times with pure water. The yield of the lyophilized magnetic nanogels was 89%.

### 2.3 Immobilization of Enzyme onto the Magnetic Nanogels with Carboxyl Groups

EDC· HCl (5 mg, 26 $\mu$mol) and NHS (7 mg, 61 $\mu$mol) were dissolved in 3 mL of phosphate buffer solution (50 mM, pH 6.5). Twenty milligrams of carboxyl-functionalized magnetic nanogels was added into the mixture. The reaction was carried out at water bath of 0°C for 30 min. The magnetic nanogels with active ester was gathered by a magnet and redispersed in 3 mL of PBS (50 mM, pH 6.5). 5 mg of CT was introduced into the former mixture, and then shaken for 24 h at room temperature. The immobilized enzyme was magnetically concentrated and washed several times with PBS (50 mM, pH 6.5) until no free enzyme was detected in the washing solution by bicinchoninic acid (BCA) protein assay. Finally, 1 mL of 10 mM ethanolamine was added into the resultant immobilized enzyme, and the reaction was kept for 1 h at room temperature. The immobilized enzyme was isolated by a magnet and redispersed in pH 3 of hydrochloric after several times washing.

### 2.4 Enzyme Activity Assay

Unit of enzyme activity (U) was defined as: 1 mg of protein will hydrolyze 1.0 $\mu$mol of BTEE per minute at pH 7.8 at 25°C.

Activity of the immobilized CT was determined by spectroscopically assayed:

$$\mathrm{BTEE} + \mathrm{H_2O} \xrightarrow{\mathrm{CT}} \mathrm{Benzoyl{-}L-Tyrosine} + \mathrm{Ethanol}$$

The assay mixture consisted of 1.42 mL of Tris-HCl buffer (80 mM, pH 7.8), 1.4 mL of 1.18 mM BTEE and 0.08 mL of 2 M $CaCl_2$). After the addition of 0.1mL of enzyme solution, the reaction was carried out at 25°C for 3 min. The suspension was immediately separated by an external magnetic field of 0.5 T and measured the absorbance of the solution at 256 nm in a UV/Vis spectrophotometer (Shimadzu, Model 1601; Tokyo, Japan). Specific activity was estimated using the following formula:

$$\text{Specific activity } [\mathrm{U/mg \cdot min}] = \Delta A/(0.964 \times \mathrm{Ew} \times 3 \times 3)$$

Where $\Delta$ A was the absorbance of the solution at 256 nm, Ew represented the amount of enzyme in 0.1 mL of enzyme solution, 0.964 was the molar extinction coefficient of *N*-Benzoyl-*L*-Tyrosine at 256 nm.

### 2.5 Effect of Temperature on the Resolution

20 mg of immobilized enzyme was dispersed in 15 mL of PBS (pH 6.8, 50 mM), and then charged into 100 mL of flask. 3.0 g of racemic phenylalanine methyl ester as a substrate was dissolved in 15 mL of toluene, and then mixed with the above enzyme solution. The reaction was kept at 25°C or 65°C of water bath for 10 h, with a shaking rate of

100 rpm/min. At a fixed time interval, the resolution product was isolated from the water phase. After completion of the hydrolysis reaction, the immobilized enzyme was magnetically concentrated and washed several times with water. The water phase was cooled at 4°C overnight, and then filtered. All the products were pooled and washed with ice water and toluene in sequence, then dried under vacuum.

### 2.6 Effect of Ionic Liquid on the Enzymatic Resolution

0.5 mmol of ionic liquid was mixed with the enzyme solution, and charged into a 100 mL flask. The composition of the organic phase was the same as above. The reaction was carried out at 25°C in a water bath for 10 h. The treatment of resolution product was the same as the method mentioned above.

### 2.7 Chiral Mobile Phase HPLC Analysis

Concentration of enantiomer from the resolution reaction was obtained by chiral mobile HPLC analysis using a reverse column with a UV detector. The mobile phase was methanol/water (v/v = 20:80) containing 1.5 mM of L-proline and 0.75 mM of copper ionic. Chromatographic studies were performed at 25°C with a flow rate of 1 mL/min. After equilibration, 5 $\mu$L of injection was made. Detection was achieved at 250 nm. Enantiomeric excess of phenylalanine (ee %) was calculated based on the HPLC analysis.



### 2.8 Reusability Assay

To test reusability of the immobilized enzyme, batch resolution reaction was conducted. After each cycle of the resolution reaction, the immobilized enzyme was isolated by a permanent magnet, and washed several times with PBS. Then, the immobilized enzyme was reintroduced into the PBS, and started a new round of resolution.

### 2.9 Characterization

The hydrodynamic sizes of magnetic nanogels were measured by dynamic light scattering (DLS) (Zetasizer Nano ZS, Malvern Instruments, UK). Thermogravimetric analysis (TG) was determined by a simultaneous DTA-TG (Shimadzu, DTG-60M) and DSC apparatus (Shimadzu, DSC-60) by heating the samples from room temperature to 800°C under $N_2$ atmosphere at a heating rate of 10°C/min. Magnetic properties of the samples were obtained with a Princeton Applied Research Vibrating Sampling Magnetometer (VSM) model 155 and a Quantum Design SQUID MPMS-XL (ac and dc modes and maximum static field 5 T).

## 3 Results and Discussion

### 3.1 Characterization of Carboxyl-Functionalized Magnetic Nanogels

To covalently immobilize the enzyme to $Fe_3O_4$ nanoparticles, magnetic nanogels covered by hydrophilic polymer containing carboxyl groups were obtained by photochemical *in situ* polymerization with quantum-sized $Fe_3O_4$ nanoparticles as photoinitiator according to our previous methods (24). The chemical structures of magnetic nanogels have been proved in our former work (24), and their mean hydrodynamic diameter measured by DLS was 45 nm.

The TG curve of magnetic nanogels was shown in Figure 1, a 1.42% weight loss of the magnetic nanogels was observed in the temperature range from room temperature to 180°C, which was the water content in magnetic nanogels. From about 180 to 480°C, an 11.98% weight loss of the magnetic nanogels showed the polymer shell content. So, the magnetic content of magnetic nanogels was calculated to be about 86.6% in the lyophilized state.

To better understand the magnetic behaviors, the hysteresis loops of magnetic nanogels were measured by VSM. As shown in Figure 2, the immeasurable coercivity and remanence of magnetic nanogels suggested that superparamagnetic properties were retained for $Fe_3O_4$ nanoparticles after surface coating. Saturation magnetization of the magnetic nanogels was measured to be 62.8 emu.g$^{-1}$, which was a slight decrease in comparison with that of naked $Fe_3O_4$ nanoparticles due to the partial oxidation of magnetite surface during the photo-polymerization process.

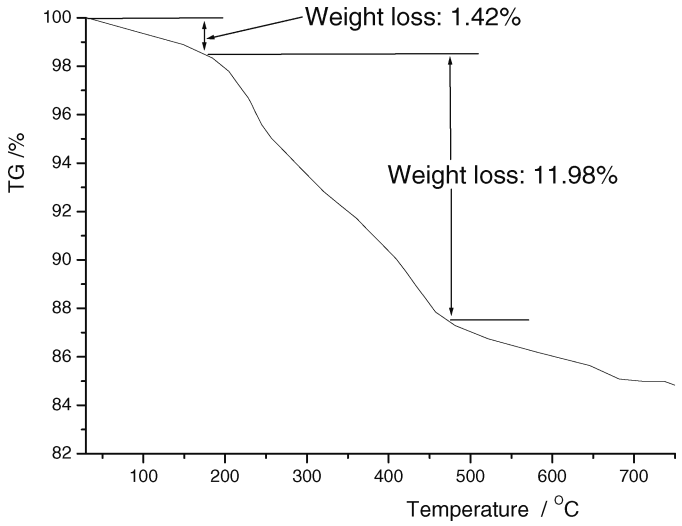



**Fig. 1.** TG curve of magnetic nanogels.

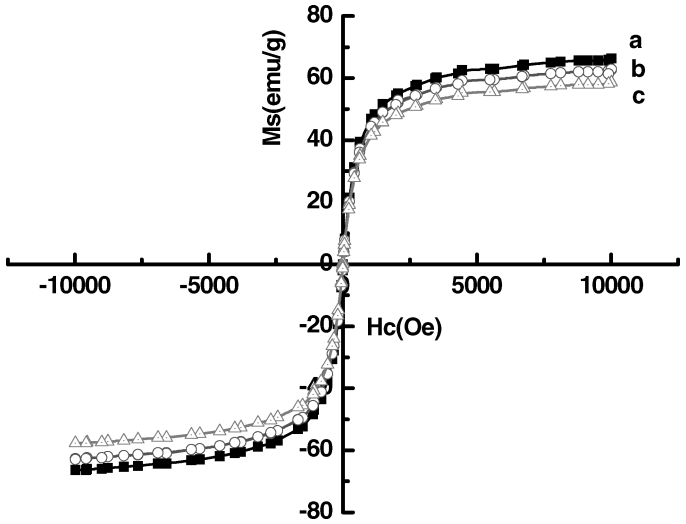



**Fig. 2.** Hysteresis loops of (a) naked $Fe_3O_4$ nanoparticles, (b) magnetic nanogels, and (c) CT-modified magnetic nanogels.

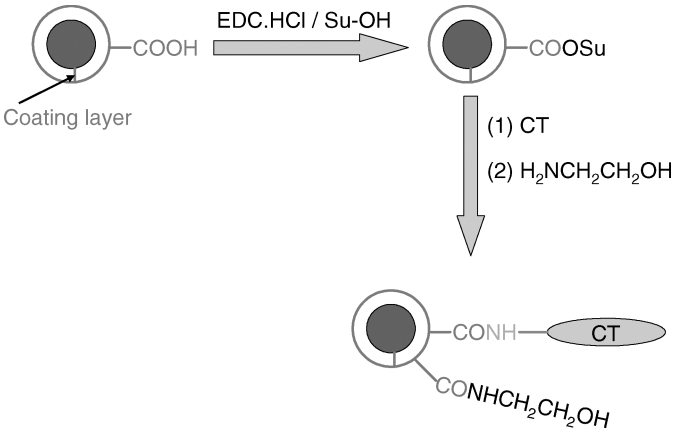



**Fig. 3.** Immobilization protocol of CT onto magnetic nanogels.

### 3.2 Characterization of Immobilized Enzyme onto the Magnetic Nanogels

$\alpha$ -Chymotrypsin (CT) was covalently bound to carboxyl-functionalized magnetic nanogels using EDC.HCl as a coupling agent. The immobilization protocol was schematically presented in Figure 3. Ethanolamine was used to block the superfluous carboxyl groups in CT-modified magnetic nanogels. The immeasurable coercivity and remanence of CT-modified magnetic nanogels also suggested their superparamagnetic behaviors (Fig. 2). Saturation magnetization of the CT-modified magnetic nanogels was measured to be 59.6 emu.$g^{-1}$. Their high saturation magnetization suggested their good magnetic recovery. The mean hydrodynamic diameter of CT-modified magnetic nanogels was measured to be 55 nm by DLS. The amount of CT on the magnetic nanogels was obtained by standard BCA protein assays of the original enzyme solution and washing solution, respectively. The binding capacity was calculated to be 37.5 mg CT/g nanogels, and activity of the immobilized CT preparation was determined to be 0.77 U/(mg. min), 82.7% as that of the free CT.

### 3.3 Effect of Temperature on the Resolution

The enzymatic resolution of (*R*, *S*)-phenylalanine was carried out in a toluene/water biphasic system. The mechanism was illustrated in Figure 4. At fixed time interval, the enzyme-catalyzed sample was separated and taken out of the reaction mixture. The optical rotation measurements and chiral mobile phase HPLC were utilized for determining the absolute configuration and enantiomeric excess (ee %) of the resolution product, respectively. As show in Table 1, a yield of 26.7% resolution was obtained for the immobilized preparation in comparison to 44.9% for the same amount of free enzyme at 25°C. The ee % values of the resolution products from the free and the immobilized enzyme were determined to be 83.5 and 91.3, respectively. The decreased resolution yield might be due to the mass transfer limitation, as well as the loss of activity after the enzyme being bound to the support. However, immobilization of enzyme to the magnetic nanogels resulted in the enhancement of the stereoselectivity of the enzyme.

As show in Table 1, when the resolution was carried out ata higher temperature of 65°C, the free enzyme lost its activity completely and no product was obtained. While the immobilized preparation gave a conversion yield of 24.0%, and a ee % of 77.1%, which was smaller than the product obtained from 25°C, in coincidence with the results previously reported (21, 25). These results indicated that the immobilized enzyme had better resistance to temperature inactivation than that of the free enzyme.

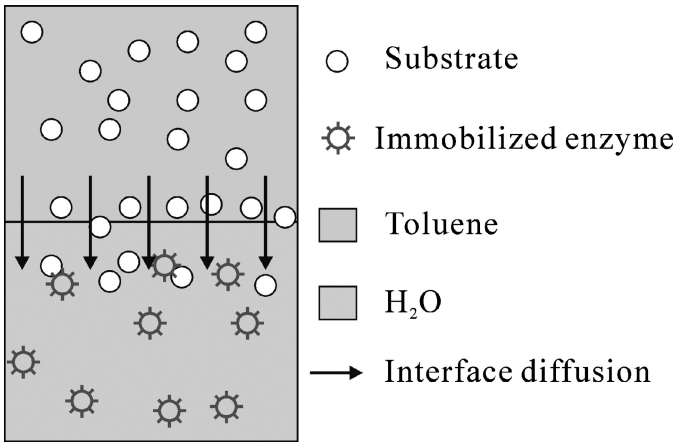
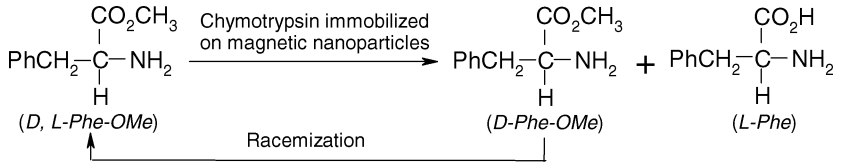



**Fig. 4.** Schematic representation of enzymatic resolution in toluene/water biphasic system.

**Table 1.** Effect of temperature or ionic liquid on the enzymatic resolution of racemic phenylalanine methyl ester




| | *Yield (%)* [a] | | *ee (%)* [a] | |
|---|---|---|---|---|
| *Entry* | *Free enzyme* | *Immobilized enzyme* | *Free enzyme* | *Immobilized enzyme* |
| 1 | 44.9 | 26.7 | 83.5 | 91.3 |
| 2 | — | 24.0 | — | 71.1 |
| 3 | 39.5 | 23.8 | 86.0 | 95.2 |

[a]Determined by Chiral mobile phase HPLC analysis.
Entry 1: Reaction carried out at 25°C;
Entry 2: Reaction carried out at 65°C;
Entry 3: Adding 0.05 mM ionic liquid to the water phase.

### 3.4 Effect of Ionic Liquid on the Enzymatic Resolution

Ionic liquid (IL) is a new generation of "green" solvent with some unique properties. It is worthwhile to find the effects of IL on the enzyme-catalyzed activity. As shown in Table 1, the addition of IL enhanced the steroselectivity of the enzyme to a certain extent while the conversion yield decreased. It was thought that the substrate turned from neutral form to ionic form after adding IL to the water phase. As a consequence, the size of the substrate became smaller. However, the size change was not big enough to lead to a structure change of the substrate (26), which reduced the probability of the enzyme molecule combining with the slow reacting enantiomer (*R*-enantiomer). Thus, steroselectivity of the immobilized enzyme in enzymatic chiral resolution was improved, while IL could enhance the ionic strength of water phase, and resulted in aggregation of immobilized enzyme. In this case, the number of enzyme molecules taking part in the hydrolysis reaction decreased, and thereby the conversion yield decreased. Detail results will be reported soon.

**Table 2.** Recycling of the immobilized enzyme from the reaction mixture for kinetic resolution of racemic phenylalanine methyl ester at 25°C, toluene/water (v/v) = 1:1

| | *Conversion (%)* [a] | |
|---|---|---|
| *Reaction cycle* | *Free enzyme* | *Immobilized enzyme* |
| 1 | 44.9 | 26.7 |
| 2 | — | 25.9 |
| 3 | — | 24.6 |

[a]Determined by chiral mobile phase HPLC analysis.

### 3.5 Recycle Usability of Immobilized CT

The recycling of the immobilized enzyme was also preliminarily investigated. After each cycle of resolution, the immobilized enzyme in water was magnetically concentrated and washed several times with PBS. Then, the immobilized enzyme was reintroduced to the reaction system, and a new cycle of resolution started. As illustrated in Table 2, the immobilized enzyme had a conversion yield of 25.9% in the second cycle of the reaction, and 24.6% in the third round. Obviously, the immobilized enzyme exhibited excellent reusability for the first several cycles.

## 4 Conclusions

In summary, magnetic nanogel-bound lipase was used to probe chiral resolution of racemic mixture in biphasic system. After bound to the support, the immobilized enzyme exhibited excellent thermal and long-term stability, yet loss in activity was also observed due to the conformational restrictions imposed by the covalent bonding of the enzyme residues to the support. The magnetic immobilized enzyme could be retrieved from the reaction mixture for a new round of resolution without significant loss in activity. The superparamagnetic feature allowed facile isolation of immobilized enzyme in a magnetic field. The ee % values of the nanoparticle-supported enzymatic resolution were enhanced to a certain extent compared to those of the free counterpart. It is important to stress that the reaction system was of high economy and efficiency. These aforementioned features of the magnetic nanogels may open a new horizon for enzymatic chiral resolution in the near future. More detailed experiments, for instance, the effects of buffer, water content and concentration of substrate on the catalytic efficiency of the immobilized

enzyme are ongoing. Relevant results will be reported in due course.

## Acknowledgements

The research work was supported by the National Natural Science Foundation of China (20504010), the 973 Projects of Chinese Ministry of Science and Technology (2009CB930000 and 2009CB930300), Shanghai Municipality Commission for Special Project of Nanometer Science and Technology (0752nm022, 0852nm03700 and 0952nm05300), and Shanghai Municipality Commission for International Corporation Project among Nongovernment (09540709000).

## References

1. Jamali, F., Mehvar, R. and Pasutto, F.M. (1989) *J. Pharm. Sci*., 78, 695.
2. Atuu, M.R., Mahmood, S.J., Laib, F. and Hossain, M.M. (2004) *Tetrahedron: Asymmetry*, 15, 3091.
3. Yamagishi, T., Miyamae, T., Yokomatsu, T. and Shibuya, S. (2004) *Tetrahedron Lett*., 45, 6713.
4. Ghanema, A. and Aboul-Eneinb, H.Y. (2004) *Tetrahedron: Asymmetry*, 15, 3331.
5. Zhai, X.R., Wei, W.Z., Zeng, J.X., Liu, X.Y. and Gong, S.G. (2006) *Anal. Lett*., 39, 913.
6. Guarise, C., Pasquato, L., De, F.V. and Scrimin, P. (2006) *Proc. Natl. Acad. Sci. USA*, 103, 3978.
7. Kim, J., Grate, J.W. and Wang, P., (2006) *Chem. Eng. Sci*., 61, 1017.
8. Bellezza, F., Cipiciani, A. and Quotadamo, M.A. (2005) *Langmuir*, 21, 11099.
9. Dyal, A., Loos, K., Noto, M., Chang, S.W., Spagnoli, C., Shafi, K.V.P.M., Ulman, A., Cowman, M. and Gross, R.A. (2003) *J. Am. Chem. Soc*., 125, 1684.
10. Kim, J. and Grate, J.W. (2003) *Nano. Lett*., 3, 1219.
11. Liao, M.H. and Chen, D.H. (2001) *Biotechnol. Lett*., 23, 1723.
12. Nishimura, K., Hasegawa, M., Ogura, Y., Nishi, T., Kataoka, K., Handa, H. and Abe, M. (2002) *J. Appl. Phys*., 91, 8555.
13. Rossi, L.M., Quach, A.D. and Rosenzweig, Z. (2004) *Anal. Bioanal. Chem*., 380, 606.
14. Horst, F., Rueda, E.H. and Ferreira, M.L. (2006) *Enzyme Microb. Technol*., 38, 1005.
15. Chen, T., Wang, L., Jiang, G.H., Wang, J.J., Wang, J.F. and Zhou, J.F., (2005) *Polymer*, 46, 5773.
16. Liu, X.Q., Guan, X.P., Xing, J.M., Ma, Z.Y. and Liu, H.H. (2003) *Chin. J. Chem. Eng*., 11, 731.
17. Chatterjee, J., Haik, Y. and Chen, C. (2001) *J. Colloid Polym. Sci*., 279, 1073.
18. Guo, Z. and Sun, Y. (2004) *Biotechnol. Prog*., 20, 500.
19. Huang, S.H., Liao, M.H. and Chen, D.H. (2003) *Biotechnol. Prog*., 19, 1095.
20. Gao, X., Yu, K.M.K., Tam, K.Y. and Tsang, S.C. (2003) *Chem. Commun*., 24, 2998.
21. Gardimalla, H.M.R., Mandal, D., Stevens, P.D., Yen, M. and Gao, Y. (2005) *Chem. Comm*., 4, 432.
22. Cao, J.Q., Wang, Y.X., Yu, J.F., Xia, J.Y., Zhang, C.F. and Yin, D.Z. (2004) *J. Magn. Magn. Mater.,* 277, 165.
23. Qu, S.C., Yang, H.B., Ren, D.W., Kan, S.H., Zou, G.T., Li, D.M. and Li, M.H. (1999) *J. Colloid. Interface Sci.*, 215, 190.
24. Hong, J., Gong, P., Xu, D., Sun, H. and Yao, S. (2007) *J. Appl. Polym. Sci*., 105, 1882.
25. Athawale, V., Manjrekar, N. and Athawale, M. (2001) *J. Mol. Catal. B-Enzym*., 16, 169.
26. Shin, J.S., Luque, S. and Klibanov, A.M. (2000) *Biotechnol. Bioeng*., 69, 577.