Chinese Science Bulletin

Application of ring-opening metathesis polymerization in study of polymer molecular weight-mediated catalytic properties of immobilized lipase

DU Chuang[1,2], ZHANG Guo[1], WANG Zhi[3], LI Lei[1], TANG Jun[1†] & WANG Lei[3†]

[1] College of Chemistry, Alan G. MacDiarmid Institute, Jilin University, Changchun, 130023, China;
[2] Key Laboratory of Excited State Physics, Changchun Institute of Optics, Fine Mechanics and Physics, Chinese Academy of Sciences, Changchun 130033, China;
[3] Key Laboratory of Molecular Enzymology and Engineering of Ministry of Education, Jilin University, Changchun 130023, China

Recently, significant efforts have been devoted into the study of the effect of hydrophobic supports on the catalytic properties of immobilized lipases. It seems that immobilization lipases on hydrophobic supports is a simple and efficient method to improve the catalytic activity of lipases. In this study, the hydrophobic poly(N-propyl-norbornene-exo-2,3-dicarboximide)s with well-controlled molecular weight were synthesized by the living ring-opening metathesis polymerization, and the lipases from *Pseudomonas sp*. were then immobilized on these hydrophobic polymer supports through the physical adsorption. The immobilized lipases exhibited higher activity and enantioselectivity for the transesterification of 2-octanol than those of free lipases. Furthermore, we investigated the polymer molecular weight-mediated catalytic properties of immobilized lipases. It was found that the catalytic activity and *E* value of the immobilized lipases increased with the increase of the polymer molecular weight. At the polymeric molecular weight of about 40kDa, the highest *E* value (58 at 54.2% of conversion, enantiomeric excess = 99%) was reached. After the molecular weight of polymers getting higher than 40 kDa, catalytic activity and *E* value of the immobilized lipase decreased.

ring-opening metathesis polymerization; interface; lipase

The adsorption or encapsulation of biocatalysts onto solid support materials has proved to be an efficient method to improve the catalytic activity of biocatalysts[1]. Lipases have gained considerable attention as versatile biocatalysts for the hydrolysis/synthesis of a wide range of esters and amides. Therefore, significant efforts have been devoted into the immobilization of lipases on suitable support materials. In general, lipases have two different structural forms. In the absence of hydrophobic supports, the active center of the lipase is secluded from the reaction medium by a polypeptide chain called "lid", which is considered as inactive "closed" form. In the presence of hydrophobic supports, however, important conformational rearrangement takes place, yielding an active "open" form of lipases, which may alter the corresponding catalytic functionality of lipases[2]. Therefore, in recent years, special emphasis has been put on to the selective adsorption of lipases on tailor-made hydrophobic support surfaces[3−6]. More recently, phospholipids analogous polymers were tethered onto the polypropylene hollow fiber microfiltration membrane to create a support for lipases immobilization[7]. The effect of the grafting degree of phospholipids analogous polymers on the catalytic properties of lipases has been reported. Petri and coworkers have also investigated the catalytic

Received July 25, 2008; accepted October 28
doi: 10.1007/s11434-008-0553-5
†Corresponding author (email: chemjtang@jlu.edu.cn, w_lei@jlu.edu.cn)
Supported by the Stake Key Development Program of Basic Research of China (Grant No.2007CB808000) and National Natural Science Foundation of China (Grant Nos. 50773028, and 20803028).

activity of lipases immobilized onto the films of cellulose esters with different side groups[8]. Up to now, however, there have been few reports on the effect of polymeric molecular weight on the catalytic properties of immobilized lipases.

One obstacle in the study of molecular weight effect is that it is very difficult to control the polymeric molecular weight precisely through the traditional polymerization methods. Living polymerization provides a new way to prepare well-defined polymers with controllable molecular weight. Compared with other living polymerization methods, ring-opening metathesis polymerization (ROMP) allows mild condition and short reaction time, thus becoming a promising method to obtain the well-defined polymers with controlled molecular weight[9]. In this study, ROMP was applied to synthesizing a series of hydrophobic polymers, poly (N-propyl-norbornene-exo-2,3-dicarboximide)s with well-controlled molecular weight. Lipases from *Pseudomonas sp.* (PSL) were then immobilized on these polymer substrates by simple physical adsorption. The immobilized lipases were used in the resolution of 2-octanol through transesterification with vinyl acetate as the acyl donor. The dependence of activity and enantioselectivity of immobilized lipase on the molecular weights of the hydrophobic polymers were investigated. To our knowledge, it is the first time to reveal the influence of molecular weight of the supporting polymers on the catalytic properties of immobilized lipases.

1 Experimental

1.1 Materials

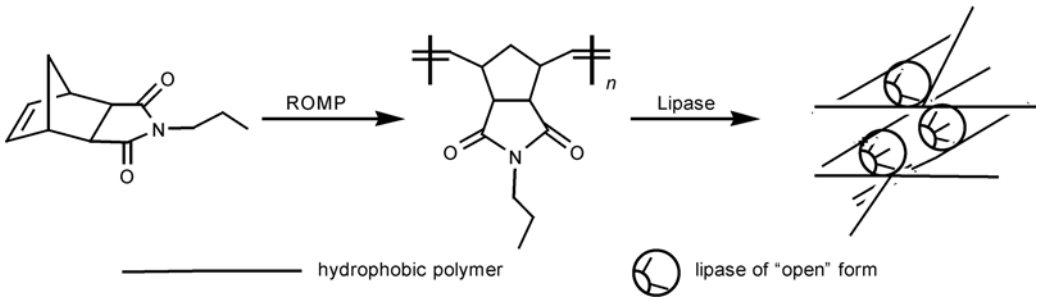
Cis-5-norbornene-exo-2,3-dicarboxylic anhydride, propylamine, triethylamine, Grubbs 2nd Catalyst [(1,3-Bis(2,4,6-trimethylphenyl)-2-imidazolidinylidene)dichloro (phenylmethylene)(tricyclohexylphosphine) ruthenium], and ethyl vinyl ether were purchased from Aldrich and used as received. Lipase from PSL was purchased from Amano Pharmaceutical Co. R-(+)-1-phenylethyl isocyanate (R-(+)-PELC) (97 %) was purchased from Fluka. All the reagents used in this study were of analytical-grade.

1.2 Preparation of N-propyl-norbornene-exo-2,3-dicarboximide

Cis-5-norbornene-exo-2,3-dicarboxylic anhydride (328 mg, 2 mmol), propylamine (124 mg, 2.1 mmol) and triethylamine (202 mg, 2 mmol) were mixed with 10 mL anhydrous toluene. The resulting mixture was allowed to reflux for 12 h. Rotary evaporation of the solvent left a yellow crude product which was purified by flash chromatography on silica gel with acetone/petrol ether (1∶4) to yield 373.2 mg (91%) of imide. ^{1}H NMR (500MHz, $CDCl_3$): δ (ppm) 6.286 (s, 2H, olefinic), 3.436 (t, 2H, J = 7.5 Hz), 3.277 (s, 2H), 2.676 (d, 2H, J = 1.0 Hz), 1.588 (dd,2H, J = 7.5, 15.0 Hz), 1.514 (1H, dd, J = 1.0, 9.5 Hz), 1.242 (1H, d, J = 9.5 Hz), 0.912 (3H, t, J = 7.5 Hz)

1.3 Preparation of poly(N-propyl-norbornene-exo-2, 3-dicarboximide)

Grubbs 2nd Catalyst (4.5 mg, 0.005mmol) was dissolved in 1.5 ml dry THF and stirred for 15 min, and then added dropwise into the THF solution containing different amounts of N-propyl-norbornene-exo-2,3-dicarboximide with vigorous stirring (from 50 to 400 equiv for each equivalent of initiator) (Scheme 1). This solution was stirred for 4 h at room temperature, and then terminated by adding ethyl vinyl ether (600 equiv). Finally, the resulting mixture was stirred for 1 h and precipitated by dropwise addition into methanol. The precipitated polymer was purified by reprecipitation from THF/methanol and dried in vacuum (85%—91% yield). ^{1}H NMR (500MHz, $CDCl_3$): δ (ppm) 5.761 (m, 1H, olefinic), 5.520 (m, 1H, olefinic), 3.340 (brs, 2H), 3.019 (m, 2H), 2.733 (m, 1H), 2.117 (m, 1H), 1.562 (br s,



Scheme 1 Procedure of the synthesis of polymers and the immobilization of lipase.

4H), 0.887 (brs, 3H).

1.4 Preparation of the enzyme solution

PSL powder (2g) was dispersed in phosphate buffer (200 ml, pH 8.0, 0.1M) at 4℃ for 2 h with stirring, and the insoluble impurity was removed by centrifugation (8000 rpm, 5 min). The supernatant was lyophilized. Enzyme solution (10 mg/mL) was prepared by dissolving the lyophilized PSL (1 g) in phosphate buffer (100 ml, pH 8.0, 0.1M).

1.5 Immobilization of lipase

50 mg poly(N-propyl-norbornene-exo-2,3-dicarboximide) was dissolved in 5 mL THF, and then mixed with 4mL enzyme solution (10 mg/mL). After vigorous stirring for 2 h, the complexes of poly(N-propyl-norbornene-exo-2, 3-dicarboximide) and lipase particles formed in the form of precipitate (Scheme 1). This precipitate was purified by filtrating and washing for several times using distilled water, and finally dried in vacuum.

1.6 Lipase catalyzed resolution of (R, S)-2-octanol.

The reaction was performed in a round bottom flask containing 2-octanol (1 mmol), vinyl acetate (5 mmol), n-hexane (10 mL), and immobilized lipase (amount of protein was 10 mg) at 30℃ for 15 h. The relative enzymatic activity (RA) was defined as a percentage of the initial transesterification rate with the immobilized lipase by comparing to the free lipase in the first 0.5 h.

1.7 Measurements

NMR spectra were conducted on a Bruker ARX-500 NMR spectrometer with $CDCl_3$ as solvent. Molecular weights and molecular weight distributions were measured on a Waters 410 gel permeation chromatography (GPC) apparatus. The 2-octanol samples were withdrawn from the vials and analyzed directly by gas chromatograph on a Shimadzu gas chromatograph (GC-14B). The enantiomeric ratio (*E* value) was determined from the degree of conversion (c) and enantiomeric excess (ee_s)[10].

2 Results and discussion

As shown in Scheme 1, the polymerization of N-propyl-norbornene-exo-2,3-dicarboximide was conducted through a ring-opening metathesis reaction catalyzed by the Grubbs 2nd Catalyst in THF. The degree of polymerization was monitored by comparing the ^{1}H NMR spectra of the monomer with the corresponding polymer (Figure 1). The evidence for the polymerization is the loss of the olefinic characteristic resonance peak at 6.2 ppm, and the appearance of resonance peaks between 5.52 and 5.76 ppm, which are the characteristics of polymers synthesized from norbornenyl-containing starting materials[11]. The feeding ratios of the monomer over initiator [M]/[I] ([M] and [I] are concentration of monomer and initiator, respective) were controlled between 50 and 400. As shown in Figure 2, in this range, a linear correlation between the polymer molecular weight and the [M]/[I] ratio was obtained. This observation is one of the features of living polymerization, since living polymerization provides a uniform rate of the increase of molecular weight[12]. As shown in Table1, the as-prepared poly(N-propyl-norbornene-exo-2,3-dicarboximide) also possesses a narrow molecular weight distribution. The PDIs of all the polymers ranged from 1.14 to 1.17. Through the variation of polymerization parameters, the molecular weights of polymers were tuned from 10 kDa to 80 kDa.

The resulting poly(N-propyl-norbornene-exo-2,3-dicarboximide)s were then conjugated with lipases by

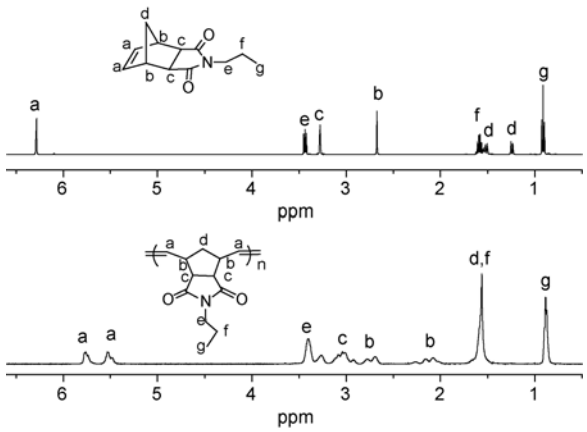


Figure 1 ^{1}H NMR of monomer (A) and polymer (B).

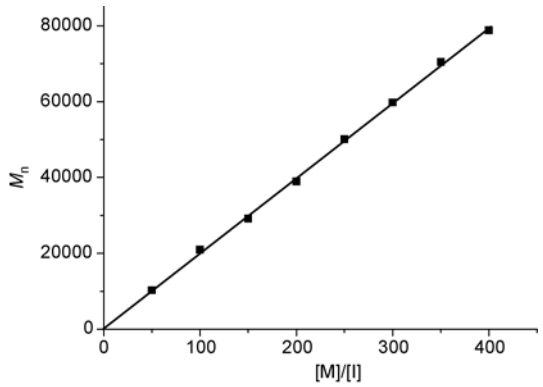


Figure 2 Dependence of the M_n on the [M]/[I] ratio for polymers.

physical adsorption (Scheme 1). The amounts of lipases immobilized on the hydrophobic poly(N-propyl-norbornene-exo-2,3-dicarboximide) were estimated by the Lowry method using bovine serium albumin (BSA) as a standard for protein concentration[13]. The amounts of lipases immobilized on these polymers were ranged from 90 to 310 mg/g (Table 1). A good immobilization protocol should keep high catalytic activity after lipase immobilization. As expected, the immobilized lipases exhibited a higher activity and enantioselectivity for the transesterification of 2-octanol than those of free lipases (Table 1). The origin of the high catalytic activity and enantioselectivity of lipases after being absorbed onto the hydrophobic polymer substrate was attributed to the shift of the conformational equilibrium of lipase towards active open form[2].

As indicated in Table 1, the molecular weight of hydrophobic polymers had a remarkable effect on the catalytic properties of immobilized lipases. The catalytic activity and *E* value of the immobilized lipases increased with the increasing of the molecular weight of polymer. When the molecular weights were above 40 kDa, catalytic activity of the immobilized lipase and *E* value decreased. This trend was attributed to the efficient contact of the active site of immobilized lipases with the substrate. With the increasing of polymeric molecular weight, the probability of the contact between substrate and immobilized lipase increased due to the hydrophobic properties of the support polymer and substrate. As a result, the catalytic activity of the immobilized lipase increased. The open form of the lipase may be fixed by the hydrophobic polymers, then the enantioselectivity of lipase could be improved simultaneously. The highest *E* value was achieved at the polymeric molecular weight of about 40 kDa. Further increasing the polymer molecular weight resulted in the decrease of the catalytic activity and enantioselectivity of the immobilized lipases. One possible reasen for the decrease was the destroying of the open form of lipase. Furthermore, the active site of lipase might be shieldeded by the overlapping of longer polymer chains, thus reducing the probability of the contact between substrate and immobilized lipase.

3 Conclusions

In summary, hydrophobic poly(N-propyl-norbornene-exo-2,3-dicarboximide)s with well-controlled molecular weight were synthesized by the living ring-opening metathesis polymerization. The lipases can then be immobilized on these hydrophilic polymer supports through the physical adsorption. The immobilized lipases exhibited a higher activity and enantioselectivity for the transesterification of 2-octanol than free PSL. The molecular weight of hydrophobic polymers had obvious effect on the catalytic properties of immobilized lipases. The highest *E* value (58 at 54.2% of conversion, enantiomeric excess = 99%) was obtained at the polymer molecular weight of about 40 kDa. These hydrophobic polymers, synthesized by the living ring-opening metathesis polymerization, are expected to improve the lipase catalytic properties in other lipases catalytic reactions as well.

Table 1 Effect of hydrophobic polymers with different molecules weight on the activity and enantioselectivity of immobilized PSL in transesterification.

[M]/[I]	$M_{n,GPC}$ a)	PDI a)	Amounts of PSL(mg/g) b)	Relative activity c)	Conversion d)	ee_s d)	*E*
–	–	–	free lipase	100%	32.3%	43.9%	37
50	10268	1.15	90	150%	38.9%	58.0%	39
100	20965	1.16	150	160%	40.7%	62.5%	41
150	29128	1.14	230	290%	48.6%	83.8%	44
200	38968	1.14	260	**342**%	**54.2**%	**99.0**%	**58**
250	50085	1.16	290	352%	53.8%	97.8%	51
300	59796	1.16	310	348%	53.0%	95.7%	47
350	70521	1.15	300	332%	52.9%	95.4%	46
400	78862	1.17	298	316%	52.7%	94.6%	44

a) THF, polystyrene standards. b) BSA standards. c) free or immobilized lipase that had the same protein content (10 mg) in the transesterification; d) conversion and ee_s value were determinated at 15 h.

1 May O, Nguyen P T, Arnold F H, Inverting enantioselectivity by directed evolution of hydantoinase for improved production of L-methionine. Nat Biotechnol, 2000, 18: 317－320
2 Mateo C, Palomo J M, Fernandez-Lorente G, et al, Improvement of enzyme activity, stability and selectivity via immobilization techniques. Enzyme Microb Tech, 2007, 40: 1451－1463
3 Reetz M T. Entrapment of biocatalysts in hydrophobic sol-gel materials for use in organic chemistry. Adv Mater, 1997, 9: 943－954
4 Fernandez-Lorente G, Palomo J M, Cabrera Z, et al. Specificity enhancement towards hydrophobic substrates by immobilization of lipases by interfacial activation on hydrophobic supports. Enzyme Microb Tech, 2007, 41: 565－569
5 Loos K, Kennedy S B, Eidelman N, et al. Combinatorial Approach To Study Enzyme/Surface Interactions. Langmuir, 2005, 21: 5237－5241
6 Edgar K J, Buchanan C M, Debenham J S, et al. Advances in cellulose ester performance and application. Prog Polym Sci, 2001, 26: 1605－1688
7 Deng H T, Xu Z K, Huang X J, et al. Adsorption and activity of *Candida rugosa* lipase on polypropylene hollow fiber membrane modified with phospholipid analogous polymers. Langmuir, 2004, 20: 10168－10173
8 Kosaka P M, Kawano Y, *El Seoud* O A, et al. Catalytic activity of lipase immobilized onto ultrathin films of cellulose esters. Langmuir, 2007, 23: 12167－12173
9 Bielawskia C W, Grubbs R H. Living ring-opening metathesis polymerization. Prog Polym Sci, 2007, 32: 1－29
10 Yu D H, Wang Z, Zhao L F, et al. Resolution of 2-octanol by SBA-15 immobilized Pseudomonas sp. Lipase. J Mol Catal B-Enzym, 2007, 48: 64－69
11 Watson K J, Zhu J, Nguyen S T, et al. Hybrid nanoparticles with block copolymer shell structures. J Am Chem Soc, 1999, 121: 462－463
12 Buchmeiser M R. Homogeneous metathesis polymerization by well-defined group VI and group VIII transition-metal alkylidenes: fundamentals and applications in the preparation of advanced materials. Chem Rev, 2000, 100: 1565－1604
13 Lowry O H, Rosebrough N J, Farr A L, et al. Protein measurement with the Folin phenol reagent. J Biol Chem, 1951, 193: 265－275

Science in China Series B: Chemistry

EDITOR-IN-CHIEF

LI Lemin
College of Chemistry and Molecular Engineering
Peking University
Beijing 100871, China

AIMS AND SCOPE

Science in China Series B: *Chemistry*, an academic journal cosponsored by the Chinese Academy of Sciences and the National Natural Science Foundation of China, and published by Science in China Press and Springer, is committed to publishing high-quality, original results in both basic and applied research.
Science in China Series B: *Chemistry* is published monthly in both print and electronic forms. It is indexed by Science Citation Index.

SUBMISSION: www.scichina.com

Orders and inquiries:
China
Science in China Press; 16 Donghuangchenggen North Street, Beijing 100717, China; Tel: +86 10 64034559 or +86 10 64034134; Fax: +86 10 64016350
North and South America
Springer New York, Inc.; Journal Fulfillment, P.O. Box 2485; Secaucus, NJ 07096 USA; Tel: 1-800-SPRINGER or 1-201-348-4033; Fax: 1-201-348-4505; Email: journals-ny@springer-sbm.com
Outside North and South America
Springer Distribution Center; Customer Service Journals; Haberstr. 7, 69126 Heidelberg, Germany; Tel: +49-6221-345-0, Fax: +49-6221-345-4229; Email: SDC-journals@springer-sbm.com