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FULL PAPER

# Covalently Immobilized Lipase on a Thermoresponsive Polymer with an Upper Critical Solution Temperature as an Efficient and Recyclable Asymmetric Catalyst in Aqueous Media

Lan-Lan Lou,[a,b] Huaxin Qu,[a] Wenjun Yu,[a] Bei Wang,[a] Lezi Ouyang,[a] Shuangxi Liu,*[a,c] and Wuzong Zhou**[b]

**Abstract:** A thermoresponsive lipase catalyst with an upper critical solution temperature (UCST) of about 26 °C was exploited by covalent immobilization of an enzyme, *Pseudomonas cepacia* lipase (PSL), onto poly(acrylamide-*co*-acrylonitrile) via glutaraldehyde coupling. The experimental conditions for the PSL immobilization were optimized. The immobilized PSL was much more stable for wide ranges of temperature and pH than the free PSL. The material was also evaluated as an asymmetric catalyst in the kinetic resolution of racemic α-methylbenzyl butyrate at 55 °C in an aqueous medium and exhibited high catalytic performance and stability. Up to 50% conversion and 99.5% product enantiomeric excess were achieved, thus providing highly pure enantiomers. More importantly, this biocatalyst could be easily recovered by simple decantation for reuse based on temperature-induced precipitation. It showed good reusability and retained 80.5% of its original activity with a well reserved enantioselectivity in the 6$^{th}$ cycle. This work would shed light on the future development of new UCST-type enzyme catalysts.

## Introduction

Owing to the increasing demands for enantioselective syntheses of fine chemicals and environmentally benign processes in the chemical industry, enzymes have recently received extensive attention as a class of highly specific, efficient and stereoselective biocatalysts.[1] Lipases are among the most useful enzymes and are widely utilized in numerous industrial biosynthetic processes for the production of optically active compounds, pharmaceuticals, agrochemicals, food, biodiesel, detergent, *etc*.[2]

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However, the free enzymes often present some disadvantages, such as low stability for long-term operation, high sensitivity towards environmental conditions, as well as difficulty in separation and recovery from the reaction system. Their practical applications are therefore severely limited. [3] Immobilization of enzymes can usually overcome these shortcomings and has thus been recognized as an appropriate method to improve the catalytic properties of enzymes.[3,4] Consequently, many efforts have been devoted to the immobilization of lipases on different solid carriers, including various inorganic materials and organic polymers, through a variety of processes, such as entrapment, physical adsorption, and covalent attachment.[5]

On the other hand, diffusional limitations of reactants to the catalytic active sites often have an adverse effect on the catalytic activity of immobilized lipases. In addition, low reusability in aqueous media is common issue over the immobilized lipases by entrapment and physical adsorption due to a large tendency for enzymes to leach from the carriers, further limiting their applications, especially for hydrolytic reactions.

In the past decades, water-soluble thermoresponsive polymers have attracted considerable interests in both academia and industry owing to their ‘‘smart’’ nature of reversible solubility changes in response to temperature,[6] which makes it highly possible to design polymer-based smart heterogeneous biocatalysts that can realize a desirable process of “homogeneous catalysis, heterogeneous recycle” by changing the system temperature. Some thermoresponsive polymers, especially polymers with a lower critical solution temperature (LCST) have been widely used as carriers for enzyme immobilization.[7] In general, the LCST-type thermoresponsive polymers, typically poly(*N*-isopropylacrylamide) and poly(*N*-vinylcaprolactam), exhibit LCSTs of about 30~35 °C, which are lower than the reaction temperature for optimum activity of most enzymes. In other words, these polymers are often insoluble and would separate from the aqueous system at the optimum temperature for enzymes, leading to unsatisfactory catalytic activity. Moreover, the catalyst separation process for a multi-cycle operation performed at elevated temperatures may always deactivate the enzymes.

In contrast, polymers with an upper critical solution temperature (UCST), above which polymers are soluble in water, display more promising potential for enzyme immobilization. As the UCST is often below the optimum temperature of enzymes, the soluble polymer molecules would remarkably reduce the mass transfer limitation for the immobilized enzymes and thus enhance the catalytic activity in such a homogeneous catalytic system.

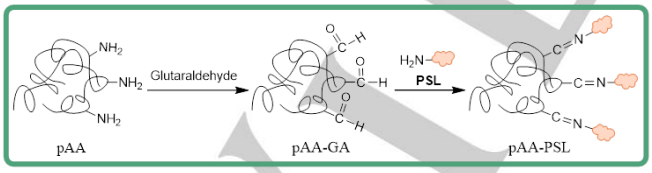
However, the reports about the immobilized enzymes with UCST-type thermoresponsive polymers to date have been still very limited. As a rare example, Limadinata *et al*. [8] reported the synthesis of UCST-type polymer nanoparticles poly(glycidyl methacrylate)/poly(acrylic acid-*co*-acrylamide) and their application as carriers to immobilize cellulase and cellobiase, also popular enzymes, for the hydrolysis of cellulose to glucose. These biocatalysts showed good catalytic performances above the UCST of 14 °C and could be easily separated from the liquid phase by cooling down the solution to a temperature below the UCST. Till now, however, the UCST-type polymers have not been investigated for their application in enantioselective biocatalysis.

In the present work, we explore immobilization of *Pseudomonas cepacia* lipase (PSL) using a UCST-type thermoresponsive polymer, poly(acrylamide-*co*-acrylonitrile) (pAA), as the carrier, and investigate its enantioselective application in an aqueous medium. PSL was covalently attached on pAA by using glutaraldehyde (GA) as a coupling agent. The produced immobilized lipase, with a UCST of about 26 °C, was applied in asymmetric hydrolysis of racemic α-methylbenzyl butyrate. The immobilization conditions, catalytic activity, enantioselectivity, stability and reusability of the immobilized enzyme are investigated in detail. This preliminary research on UCST-type thermoresponsive lipase catalyst may shed light on the future development of new enzyme catalysts.

## Results and Discussion

### Synthesis and turbidity measurement of immobilized PSL on pAA

The polymer pAA was synthesised through the polymerization of acrylamide and acrylonitrile with a molar ratio of 82 : 18 by using 2,2-azobisisobutyronitrile (AIBN) as an initiator.[9] Enzyme PSL was then covalently immobilized on pAA by using GA, in which the aldehyde groups reacted with the amino groups in the polymer as well as in the PSL molecules (Scheme 1). The resulting specimen was denoted as pAA-PSL.




**Scheme 1.** Glutaraldehyde-mediated covalent immobilization of PSL on the polymer pAA.

The thermoresponsive behaviour of pAA is important as the activity of enzyme immobilized on it can be adjusted by changing the temperature above and below the UCST. To characterize the thermoresponsive behaviours of pAA and pAA-PSL, turbidity observation was carried out by measuring the 500 nm UV transmittance of these polymer samples with a total concentration of 0.5 wt% in a phosphate buffer solution (pH 7.0, 0.05 M).

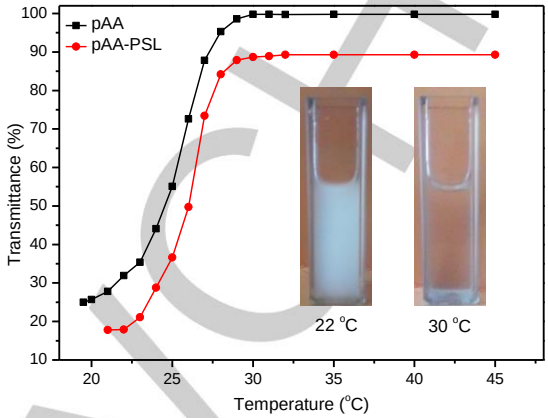



**Figure 1.** Turbidity curves of 0.5 wt% of pAA and pAA-PSL in a phosphate buffer solution (pH 7.0, 0.05 M) and photographs of pAA-PSL in the phosphate buffer solution at 22 °C and 30 °C (inset).

As seen in Figure 1, the polymer pAA shows a UCST of about 26 °C. This phase transition behaviour can be mainly attributed to the increased inter-/intra-chain hydrogen bonding interaction (C=O···H-N) in the polymer molecules upon cooling.[10] The immobilized enzyme pAA-PSL also exhibits a thermoresponsive behaviour with a very similar UCST to the pAA carrier, although the overall transmittance of the former is about 10% lower than the latter, indicating that incorporation with the enzyme reduces the solubility of the polymer. Consequently, the phosphate buffer solution of pAA-PSL is cloudy at 22 °C and becomes clear at 30 °C. This UCST-behaviour is highly desirable since the reaction temperature for an optimum activity of most enzymes is higher than this UCST. Thus efficient catalysis can be expected above the UCST over the immobilized enzymes that are well dissolved, and separation of the catalysts can be easily achieved by precipitation below the UCST.

### Optimization of conditions for PSL immobilization

The experimental conditions for the PSL immobilization were optimized by investigating the effects of the GA concentration, PSL concentration, and immobilization time on the enzyme loading and specific activity measured in tributyrin hydrolysis of the immobilized PSL.

The effects of the GA concentration on the enzyme loading and specific activity of pAA-PSL were studied in the range of 0.03 mol $L^{-1}$ to 0.19 mol $L^{-1}$ at 45 °C, and the results are depicted in Figure 2a. It is observed that the enzyme loading increases notably with the increase of the GA concentration from 0.03 to 0.08 mol $L^{-1}$, and then reaches a steady stage between 0.08 and 0.13 mol $L^{-1}$, followed by a sharp decrease with further raising the GA concentration. The specific activity of immobilized PSL exhibits a

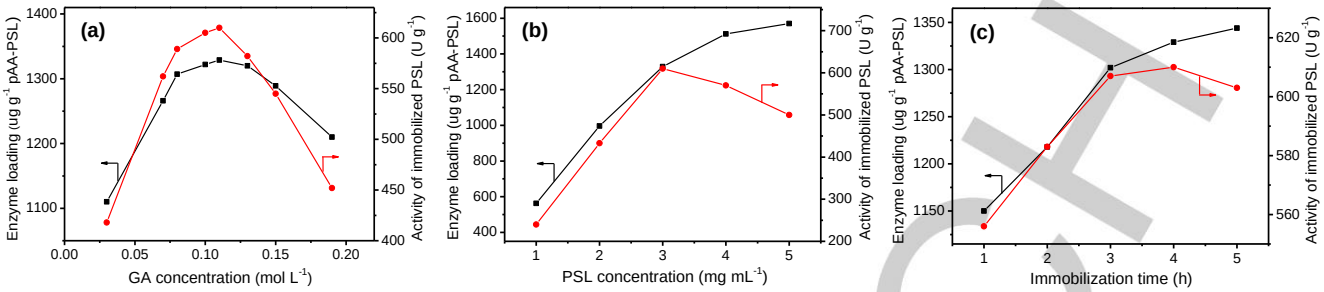



**Figure 2.** Effects of the GA concentration (a), the PSL concentration (b), and immobilization time (c) on enzyme loading and specific activity of the immobilized PSL.

similar trend to that of enzyme loading, and the highest value is obtained at the GA concentration of 0.11 mol $L^{-1}$.

This phenomenon is well consistent with the previous reports.[11] It was reported that the GA concentration has a noticeable effect on its own structure and the activity of immobilized enzyme. With an appropriate GA concentration, most GA molecules link the polymer support and PSL molecules with a dimer form, which is beneficial to reach a high activity for immobilized enzyme by exposing sufficiently the active sites on the enzyme. Whereas GA exists mainly in a monomeric or polymeric form when a lower or higher GA concentration is adopted. In both cases, the enzyme PSL is apt to closely attach on the polymer carrier, which is unfavorable for the access of the substrate to the active sites of enzymes, thus leading to an inferior activity.

The influence of the PSL concentration on the enzyme loading and specific activity of the immobilized PSL was studied in a range of 1 to 5 mg $mL^{-1}$. The results are presented in Figure 2b. A significant dependence of enzyme loading as well as the activity of immobilized PSL on the PSL concentration can be found. The enzyme loading monotonically increases with increasing the PSL concentration.

As for the specific activity of the immobilized PSL, a remarkable ascent is observed with increasing the PSL concentration up to 3 mg $mL^{-1}$, where a maximum value of 610 U $g^{-1}$ is reached, followed by a gradual decline with further increase of the PSL concentration from 3 to 5 mg $mL^{-1}$. The decreased activity of immobilized PSL at high PSL concentration may be ascribed to the increased intermolecular reaction of the immobilized lipase with a relatively high enzyme loading, when the enzyme undergoes a multilayer aggregation on the polymer.[7b,12] With the decrease in the enzyme loading, on the other hand, a monolayer of the immobilized enzyme may be presented on the polymer support,[13] which is favorable to the maintenance of the active conformation of the PSL molecules as well as to the accessibility of the active sites of enzyme by the substrate. In consequence, the maximum activity of the immobilized PSL can be achieved.

The influence of immobilization time on the enzyme loading and specific activity was investigated in a range of 1 ~ 5 h. The results, depicted in Figure 2c, show that the enzyme loading increases monotonically with the increase of immobilization time, while the specific activity of immobilized PSL shows the best result when the immobilization time is about 4 h. At this point, the enzyme loading is 1.33 mg $g^{-1}$ pAA-PSL and the specific activity of immobilized PSL is 610 U $g^{-1}$ pAA-PSL. With prolonging the immobilization time, the overloaded PSL molecules may undergo multilayer adsorption, which can not only change the conformation of enzyme molecules, but also make some of the enzyme molecules inaccessible by the substrate.[12]

This optimum enzyme loading, however, is at a relatively low level as compared with those for other immobilized biocatalysts reported in the literature.[11b,13-16] Increase of the added enzyme concentration made an insignificant change in the enzyme loading. The low loading may be resulted from the inferior reaction efficiency between the polymer and GA as well as from the low enzyme content in the PSL reagent.

### Activity and stability of immobilized PSL

The activity of pAA-PSL for tributyrin hydrolysis was studied at different temperatures and pH values. For comparison, free PSL was also tested under the same reaction conditions. The influence of temperature on hydrolytic activity of pAA-PSL and free PSL was investigated in a range of 40-65 °C with pH of 7.0. The results are given in Figure 3a. It can be found that both pAA-PSL and free PSL give the highest activity at 55 °C. At this point, pAA-PSL exhibits a specific activity of 650 U $g^{-1}$ pAA-PSL, namely 489 U $mg^{-1}$ protein, suggesting a high retainment of ~70% of the free PSL activity, 693 U $mg^{-1}$ protein. In addition, at other temperatures below or above 55 °C, remarkably more stable activities are achieved over immobilized PSL compared with the free PSL, suggesting the stabilization of PSL activity upon immobilization on the polymer. The improvement in stability of the enzyme activity can be mainly attributed to the multipoint attachment between the polymer pAA and the PSL molecules, which can effectively prevent the interaction between enzyme molecules as well as thermal denaturation of PSL.

Figure 3b shows the influence of pH value on hydrolytic activity of pAA-PSL and free PSL in a range of 6.0-8.0 with a constant temperature of 55 °C. The optimum pH value is determined to be 7.0 for both pAA-PSL and free PSL. Apart from this, compared with free PSL, highly stable activities of above 90% were also achieved over the immobilized PSL at other pH values. For free PSL, however, notably decreases in activities were observed

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above or below 7.0, especially, a low relative activity of 48.5% was obtained at pH 8.0. All the results indicate that the immobilization of PSL onto pAA effectively enhances the stability of the enzyme activity towards variations of temperature and pH.

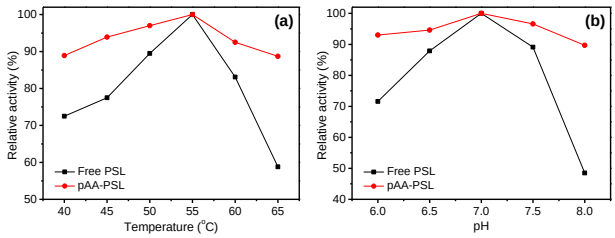



**Figure 3.** Effects of temperature (a) and pH (b) on hydrolytic activity of free and immobilized PSL.

In order to get further insight into the thermal stability of the immobilized enzyme, pAA-PSL as well as free PSL was incubated at 60 °C for various periods and then the residual activities were measured. As shown in Figure 4, the immobilized PSL exhibits a superior thermostability compared with the free PSL, e.g. they retain 97.7% and 73.3% respectively of the original activity after 1 h of incubation. With increasing the incubation time, the residual activities of both the enzyme samples decrease, whereas the immobilized PSL shows a slower descending tendency than free PSL. For example, after 12 h of incubation, free PSL only presents a residual activity of 34.4%, while pAA-PSL still maintains 68.8% of its original activity. These results suggest that the thermal stability of PSL is significantly enhanced upon immobilization onto pAA, which is very favorable from the point of view of practical application.

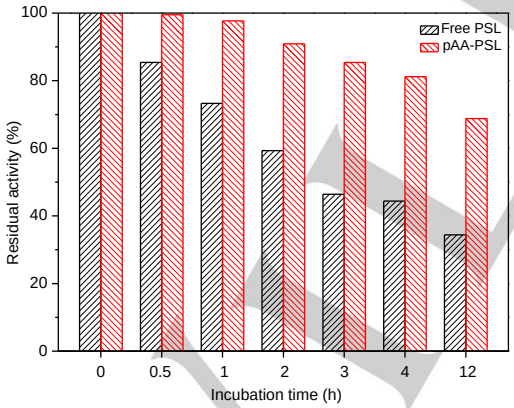
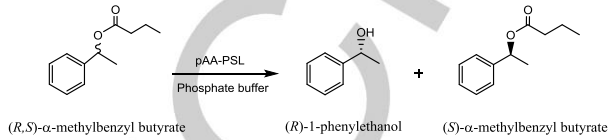



**Figure 4.** Thermal stability of immobilized and free PSL at 60 °C for 12 h.

### Kinetic resolution of (*R*,*S*)-α-methylbenzyl butyrate catalyzed by immobilized PSL in aqueous media

The immobilized enzyme pAA-PSL was evaluated as a soluble catalyst in kinetic resolution of (*R*,*S*)-α-methylbenzyl butyrate to produce (*R*)-1-phenylethanol in aqueous media at 55 °C (Scheme 2). For comparison, the free PSL with the same protein content was also examined under the identical reaction conditions. Blank experiment showed that no reaction occurred in the absence of PSL. Table 1 gives the conversions and enantioselectivities of the enzymatic kinetic resolution at different reaction time and substrate concentration using pAA-PSL and free PSL.




**Scheme 2.** The chemical reaction of kinetic resolution of (*R*,*S*)-α-methylbenzyl butyrate to produce (*R*)-1-phenylethanol using immobilized enzyme pAA-PSL as catalyst.

With 0.05 M of racemic α-methylbenzyl butyrate, pAA-PSL exhibits 96.3% of the free enzyme activity at 6 h (33.8/35.1, Table 1, entry 2) and furnishes higher conversions after 9 h compared with free PSL. 50.0% conversion is achieved over pAA-PSL at 15 h, whereas the free PSL shows a conversion of 45.9% under the same conditions (Table 1, entry 5). In addition, the immobilized PSL shows high enantioselectivity throughout the reaction with the product enantiomeric excesses ($ee_p$) above 98.9% and E > 200. These results indicate that pAA-PSL possesses high catalytic performance and that (*R*)-α-methylbenzyl butyrate is almost completely hydrolysed at 15 h to produce highly pure enantiomers. The higher conversion over pAA-PSL compared to free enzyme can be attributed to the enhancement of enzyme stability through immobilization.

With the increase of substrate concentration, a gradual decrease in the conversion was observed for both the immobilized and free enzymes. The conversions were reduced to 46.5% and 41.1% for pAA-PSL and free PSL, respectively, at 15 h of reaction as the substrate concentration was raised to 0.15 M (Table 1, entry 8). Further increasing the substrate concentration to 0.25 M, only 22.3% and 17.2% conversions over pAA-PSL and free PSL respectively were obtained at the same reaction time (Table 1, entry 11). It is noted that in all cases higher conversions were achieved over immobilized enzyme at extended reaction time compared to the free enzyme, confirming the better stability of immobilized enzyme.

As for the enantioselectivity, a slight declining trend was detected for the two enzyme catalysts when the substrate concentration was increased. For instance, the $ee_p$ and the E value over pAA-PSL at 3 h of the reaction decreased from 98.9% and >200 to 97.1% and 74.4, respectively, with the substrate concentration increased from 0.05 M to 0.25 M (Table 1, entry 1 versus 9).

The reusability of UCST-biocatalyst pAA-PSL was studied in the kinetic resolution of (*R*,*S*)-α-methylbenzyl butyrate. Each experiment was carried out in a phosphate buffer solution (2.5 mL, pH 7.0, 0.05 M) at 55 °C for 12 h with 0.125 mmol of fresh substrate. After each reaction, the catalyst was easily precipitated by cooling the reaction mixture below the UCST (Figure 5a). The

**Table 1.** Kinetic resolution of (*R*,*S*)-α-methylbenzyl butyrate catalyzed by pAA-PSL and free PSL[a]

| Entry | $C_s$ (M)[b] | Time (h) | Conv (%)[c] | | $ee_p$ (%)[d] | | E[e] | |
|---|---|---|---|---|---|---|---|---|
| | | | Free PSL | pAA-PSL | Free PSL | pAA-PSL | Free PSL | pAA-PSL |
| 1 | 0.05 | 3 | 25.9 | 21.9 | 98.7 | 98.9 | >200 | >200 |
| 2 | | 6 | 35.1 | 33.8 | 99.0 | 99.1 | >200 | >200 |
| 3 | | 9 | 39.8 | 42.9 | 99.3 | 99.3 | >200 | >200 |
| 4 | | 12 | 43.3 | 48.9 | 99.5 | 99.5 | >200 | >200 |
| 5 | | 15 | 45.9 | 50.0 | 99.5 | 99.5 | >200 | >200 |
| 6 | 0.15 | 3 | 16.9 | 14.8 | 97.3 | 97.8 | 88.8 | 106.3 |
| 7 | | 9 | 32.2 | 33.8 | 99.1 | 99.0 | >200 | >200 |
| 8 | | 15 | 41.1 | 46.5 | 99.3 | 99.5 | >200 | >200 |
| 9 | 0.25 | 3 | 6.3 | 8.8 | 97.2 | 97.1 | 73.7 | 74.4 |
| 10 | | 9 | 13.7 | 18.8 | 98.1 | 98.8 | 121.8 | >200 |
| 11 | | 15 | 17.2 | 22.3 | 98.5 | 99.0 | 160.7 | >200 |

[a] Reaction condition: 10 mg pAA-PSL or 1.6 mg free PSL, 2.5 mL phosphate buffer solution (pH 7.0, 0.05 M), 55 °C. [b] $C_s$ represents the substrate concentration in the reaction system. [c] Conv (%) represents the substrate conversion and is determined by GC with a RESTEK RT-BetaDEXse chiral column. [d] $ee_p$ (%) represents the enantiomeric excess of alcohol product and is determined by GC with a RESTEK RT-BetaDEXse chiral column. [e] E represents the enantiomeric ratio of the reaction and is calculated by the expression ln[1-Conv(1+$ee_p$)]/ln[1-Conv(1-$ee_p$)].

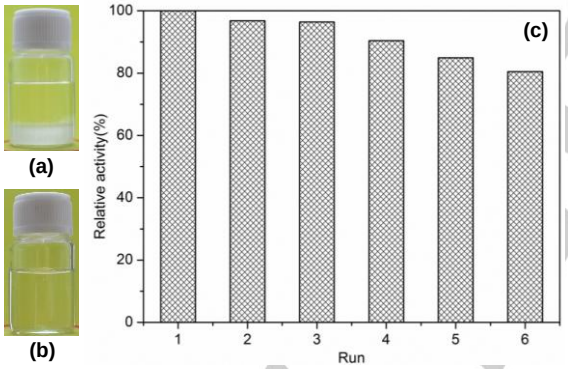



**Figure 5.** Photographs of catalytic reaction solutions after cooling to 0 °C (a) and under a new cycle at 55 °C (b). (c) Reusability of pAA-PSL in the kinetic resolution of (*R*,*S*)-α-methylbenzyl butyrate. Relative activities were calculated by taking the initial activity for the first time as 100%.

catalyst recovered via simple decantation and washing was then well redispersed in the phosphate buffer solution for next cycle (Figure 5b). The Bradford assay showed that there was no detectable protein leaching in the supernatant and washings after each cycle, indicating that the enzyme PSL was strongly attached on the polymer.

The recycling results are depicted in Figure 5c. It shows that pAA-PSL maintains a high catalytic activity in the third cycle and furnishes 96.4% of its initial activity. After six cycles, the catalyst still remains 80.5% of its original activity. In addition, the enantioselectivity of the reaction is well reserved (99.5% $ee_p$ and E > 200) during the recycling experiments. According to the previous researches, it is quite common for immobilized enzymes to drop their catalytic activity after several runs. For example, three lipases covalently immobilized on polymer-encapsulated magnetic nanobeads retained about 40~90% of the initial activity after six cycles in the hydrolysis of *rac*-α-methylbenzyl butyrate in aqueous solutions.[14] Lipase from *Burkholderia cepacia* immobilized into calcium carbonate microcapsule showed about 47% of its original activity after five cycles in the kinetic resolution of *rac*-2-acetoxyhexyl tosylate in an aqueous phase.[15] 65% of the initial activity was kept after ten cycles in the hydrolysis of olive oil by *Candida rugosa* lipase covalently immobilized on core/shell magnetic composite microspheres.[16] Han *et al*. reported that the immobilized *Pseudomonas fluorescens* lipase on porous polyuria remained 73% of its initial activity after five cycles in the hydrolysis of *p*-nitrophenyl palmitate.[11b] The recycling experiments in the present work demonstrate that the new immobilized biocatalyst has high stability and durability for the kinetic resolution reaction. The obtained result is among the best results with regard to the reusability for the immobilized lipase catalysts towards hydrolytic reactions.

## Conclusions

The thermoresponsive polymer pAA with a UCST of 26 °C was synthesised, surface-modified with GA and then applied to covalently immobilize an enzyme of PSL to afford a biocatalyst with high catalytic performance and stability. The optimal experimental conditions for PSL immobilization, including GA concentration, PSL concentration and immobilization time, were determined, under which the highest activity of 650 U $g^{-1}$ was achieved over immobilized PSL with an enzyme loading of 1.33 mg $g^{-1}$ pAA-PSL, suggesting a high specific activity retention of about 70% compared with the free enzyme. Upon immobilization, significantly enhanced stability of enzyme activity was obtained towards different temperature and pH. After 12 h of incubation at 60 °C, the immobilized PSL still maintained 68.8% of its original activity comparing with only 34.4% for free PSL. The immobilized biocatalyst pAA-PSL, with a similar UCST to the carrier, was evaluated in the kinetic resolution of (*R*,*S*)-α-methylbenzyl butyrate in aqueous media. Higher catalytic performance than the free PSL was achieved over pAA-PSL at 55 °C with conversion and $ee_p$ values of up to 50% and 99.5%, respectively, giving highly pure enantiomers. Apart from this, the immobilized biocatalyst was easily separated and recovered from the reaction system at 0 °C and showed good durability retaining 80.5% of its original activity with a well reserved enantioselectivity after six cycles. These results indicate that the UCST-type thermoresponsive polymer pAA is a promising carrier for enzyme immobilization and application.

## Experimental Section

### General

Acrylonitrile (99%, Aladdin Ind. Corp.) was purified by 8% NaOH before use. Acrylamide (99%, Energy Chemical) was recrystallized from acetone. AIBN (AR, Wako Pure Chemical Ind., Ltd.) was recrystallized from ethanol and dried under vacuum. Tributyrin (98%, Energy Chemical), GA (50 wt % in water, Aladdin Ind. Corp.), (*R*,*S*)-α-methylbenzyl butyrate (99%, Damas-beta), and PSL ("Amamo Enzyme", Novocata) were used as received. The protein content of PSL was determined to be 0.83 wt %.

The $^1$H NMR spectrum was recorded at 300 MHz using a Varian Mercury Vx-300 spectrometer. The FT-IR spectrum was taken in the transmission mode by using a Bruker Tensor 27 FT-IR instrument. The turbidity measurements were carried out on a Shimadzu UV-2550 spectrophotometer at 500 nm with deionized water as a reference (100% transmittance). Temperature was controlled by a Shimadzu TCC-240A temperature controller. Before each measurement, the temperature point was held for 2 min to ensure the thermal equilibrium of the sample cell.

Conversion and enantioselectivity were determined by gas chromatography (GC) equipped with a flame ionization detector and a RESTEK RT-BetaDEXse chiral capillary column (30 m × 0.25 mm × 0.25 μm). The column temperature was set at 120 °C. The substrate conversion was expressed as Conv (%) =100 × [1- ($S_1$ + $R_1$)/($S_0$ + $R_0$)], where $S_0$ and $R_0$ are the concentrations of (*S*)- and (*R*)-α-methylbenzyl butyrate before reaction, $S_1$ and $R_1$ are the concentrations of (*S*)- and (*R*)-α-methylbenzyl butyrate after reaction, respectively. The $ee_p$ was defined as $ee_p$ (%) =100 × ($R_2$ – $S_2$)/($S_2$ + $R_2$), where $S_2$ and $R_2$ are the product concentrations of (*S*)- and (*R*)-1-phenylethanol after reaction, respectively. The enantiomeric ratio of the reaction (*E*) was calculated using ln[1-Conv(1+$ee_p$)]/ln[1-Conv(1-$ee_p$)] as described by Chen *et al*.[17]

### Synthesis of polymer pAA

The UCST-thermoresponsive polymer pAA was prepared according to a literature method [9] with a slight modification. In a 25 mL round-bottom flask, acrylonitrile (1.08 mmol) and acrylamide (4.92 mmol) were dissolved in distilled dimethyl sulfoxide (DMSO, 5 mL), followed by an addition of AIBN (0.03 mmol) at room temperature. The flask was then placed into an oil bath for polymerization at 65 °C for 6 h under nitrogen protection. After that, the reaction mixture was quickly cooled to room temperature in an ice-water bath, and the polymer was precipitated in excess methanol and isolated by filtration. The obtained crude polymer was further purified by washing with methanol thrice, and then dried in vacuum at 70 °C for 24 h. $^1$H NMR (DMSO-d6, 300 MHz): $\delta$ (ppm) 1.2-1.9 (polymer backbone, –C$H_2$–), 2.0-2.7 (polymer backbone, –C$H$–CON$H_2$), 2.3-3.0 (polymer backbone, –C$H$–CN), 6.8 (–N$H_2$). FT-IR: 3420 ($\nu_{NH}$), 2930 ($\nu_{CH}$), 2242 ($\nu_{C\equiv N}$), 1669 ($\nu_{C=O}$) $cm^{-1}$.

### Immobilization of PSL on pAA

Enzyme PSL was immobilized on the polymer pAA via covalent bonding. To accomplish this, pAA was first modified with aldehyde functional groups through reacting with GA.[18] In a typical process, pAA (50 mg) and GA (9 ~ 57 mg) were stirred in deionized water (3 mL) at 45 °C for 6 h. Then the modified polymer, denoted as pAA-GA, was separated by cooling precipitation and rinsed thoroughly with deionized water to remove unreacted GA.

Thereafter, the immobilization of PSL was performed as follows. PSL (3 ~ 15 mg) was dissolved in a phosphate buffer solution (3 mL, pH 7.0, 0.05 M), to this solution pAA-GA (50 mg) was added, and the mixture was stirred at 45 °C for 1 ~ 5 h. After the reaction mixture was cooled in an ice-water bath, the immobilized enzyme pAA-PSL was separated by decantation, washed twice with the phosphate buffer solution (1 mL), and then freeze-dried. The protein concentration in the supernatant and washings was determined by the Bradford assay using bovine serum albumin as a standard.[19] The enzyme loading of immobilized PSL, which was defined as the amount of the protein bound per gram of pAA-PSL, was thus calculated by subtracting the residual amount of the protein after immobilization from the initial protein content.

### Determination of the activity and thermal stability of PSL

The specific activity of enzyme PSL was measured by the hydrolysis of tributyrin as follows. Typically, immobilized PSL (10 mg) or free PSL (2 mg) was mixed with a phosphate buffer solution (2 mL, pH 7.0, 0.05 M) at 45 °C under stirring for 2 min, followed by addition of tributyrin (20 μL). The mixture was stirred for another 15 min. After that, methanol (10 mL) was added to terminate the reaction. The yield of butyrate was measured by titration with a sodium hydroxide solution (0.05 M). The specific activity of enzyme PSL was described as U per g of catalyst, where U represents the enzyme unit and one U is defined as the amount of PSL required to release 1 μmol of butyrate per minute under the assay conditions.

To study the thermal stability of enzyme PSL, the immobilized PSL or free PSL in the phosphate buffer solution was incubated in a water bath of 60 °C for 0.5 ~ 12 h. The remaining activity was measured by hydrolysis

of tributyrin at 45 °C as described above. The initial activity without the thermal incubation was preset to be 100%.

#### Kinetic resolution of racemic α-methylbenzyl butyrate

The immobilized lipase pAA-PSL was evaluated as an enantioselective catalyst in the kinetic resolution reaction of racemic α-methylbenzyl butyrate. For comparison, the free enzyme PSL was also examined. A typical procedure for the enzyme-catalyzed kinetic resolution reaction was as follows. (*R*,*S*)-α-methylbenzyl butyrate (0.125 mmol) was dispersed in a phosphate buffer solution (2.5 mL, pH 7.0, 0.05 M), to which was added pAA-PSL (10 mg) or 1.6 mg of free PSL. The mixture was stirred at 55 °C for 3 ~ 15 h. After that, the mixture was cooled in an ice-water bath and the precipitated catalyst was separated by simple decantation, washed thoroughly with the phosphate buffer solution, and then submitted to the next cycle. The resulting supernatant together with the washings was subjected to GC analysis.

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[1] a) S. J. Benkovic, S. Hammes-Schiffer, *Science* **2003**, *301*, 1196–1202; b) C. M. Clouthier, J. N. Pelletier, *Chem. Soc. Rev.* **2012**, *41*, 1585–1605.

[2] P.-Y. Stergiou, A. Foukis, M. Filippou, M. Koukouritaki, M. Parapouli, L. G. Theodorou, E. Hatziloukas, A. Afendra, A. Pandey, E. M. Papamichael, *Biotechnol. Adv.* **2013**, *31*, 1846–1859.

[3] R. A. Sheldon, S. van Pelt, *Chem. Soc. Rev.* **2013**, *42*, 6223–6235.

[4] a) D. N. Tran, K. J. Balkus Jr., *ACS Catal.* **2011**, *1*, 956–968; b) R. C. Rodrigues, C. Ortiz, Á. Berenguer-Murcia, R. Torres, R. Fernández-Lafuente, *Chem. Soc. Rev.* **2013**, *42*, 6290–6307; c) P. Adlercreutz, *Chem. Soc. Rev.* **2013**, *42*, 6406–6436.

[5] a) Z. Zhou, M. Hartmann, *Chem. Soc. Rev.* **2013**, *42*, 3894–3912; b) W. Feng, P. Ji, *Biotechnol. Adv.* **2011**, *29*, 889–895; c) S. A. Ansari, Q. Husain, *Biotechnol. Adv.* **2012**, *30*, 512–523; d) J. Liu, Q. Yang, C. Li, *Chem. Commun.* **2015**, *51*, 13731–13739; e) X. Wu, M. Hou, J. Ge, *Catal. Sci. Technol.* **2015**, *5*, 5077–5085.

[6] a) M. A. C. Stuart, W. T. S. Huck, J. Genzer, M. Müller, C. Ober, M. Stamm, G. B. Sukhorukov, I. Szleifer, V. V. Tsukruk, M. Urban, F. Winnik, S. Zauscher, I. Luzinov, S. Minko, *Nat. Mater.* **2010**, *9*, 101–113; b) J. Zhuang, M. R. Gordon, J. Ventura, L. Li, S. Thayumanavan, *Chem. Soc. Rev.* **2013**, *42*, 7421–7435; c) J. P. A. Custers, S. F. G. M. van Nispen, A. Can, V. R. de La Rosa, S. Maji, U. S. Schubert, J. T. F. Keurentjes, R. Hoogenboom, *Angew. Chem. Int. Ed.* **2015**, *54*, 14085–14089; *Angew. Chem.* **2015**, *127*, 14291–14295.

[7] a) B. Trzebicka, R. Szweda, D. Kosowski, D. Szweda, Ł. Otulakowski, E. Haladjova, A. Dworak, *Prog. Polym. Sci.* **2017**, *68*, 35–76; b) H. Chen, L.-H. Liu, L.-S. Wang, C.-B. Ching, H.-W. Yu, Y.-Y. Yang, *Adv. Funct. Mater.* **2008**, *18*, 95–102; c) K. J. Mackenzie, M. B. Francis, *J. Am. Chem. Soc.* **2013**, *135*, 293–300; d) Q. Wu, T. Su, Y. Mao, Q. Wang, *Chem. Commun.* **2013**, *49*, 11299–11301.

[8] P. A. Limadinata, A. Li, Z. Li, *Green Chem.* **2015**, *17*, 1194–1203.

[9] J. Seuring, S. Agarwal, *Macromolecules* **2012**, *45*, 3910–3918.

[10] L. Hou, P. Wu, *Soft Matter* **2015**, *11*, 7059–7065.

[11] a) L. Betancor, F. López-Gallego, A. Hidalgo, N. Alonso-Morales, G. D.-O. C. Mateo, R. Fernández-Lafuente, J. M. Guisán, *Enzyme Microb. Tech.,* **2006**, *39*, 877–882; b) H. Han, Y. Zhou, S. Li, Y. Wang, X. Z. Kong, *ACS Appl. Mater. Interfaces* **2016**, *8*, 25714–25724.

[12] E. Lai; Y. Wang; Y. Wei, G. Li, G. Ma, *J. Appl. Polym. Sci.* **2016**, *133*, 43343–43351.

[13] G. Peng, C. Zhao, B. Liu, F. Ye, H. Jiang, *Appl. Surf. Sci.* **2012**, *258*, 5543–5552.

[14] Y. K. Sim, S. Jung, J. Y. Lim, J. Kim, S.-H. Kim, B. K. Song, B. T. Kim, H. Lee, S. Park, *Tetrahedron Lett.* **2011**, *52*, 1041–1043.

[15] M. Fujiwara, K. Shiokawa, K. Yotsuya, K. Matsumoto, *J. Mol. Catal. B: Enzym.* **2014**, *109*, 94–100.

[16] L. Fan, B. Zhang, H. Zhang, X. Jia, X. Chen, Q. Zhang, *RSC Adv.* **2016**, *6*, 65911–65920.

[17] C.-S. Chen, Y. Fujimoto, G. Girdaukas, C. J. Sih, *J. Am. Chem. Soc.* **1982**, *104*, 7294–7299.

[18] A. Hamerska-Dudra, J. Bryjak, A. W. Trochimczuk, *Enzyme Microb. Tech.* **2007**, *41*, 197–204.

[19] M. M. Bradford, *Anal. Biochem.* **1976**, *72*, 248–254.

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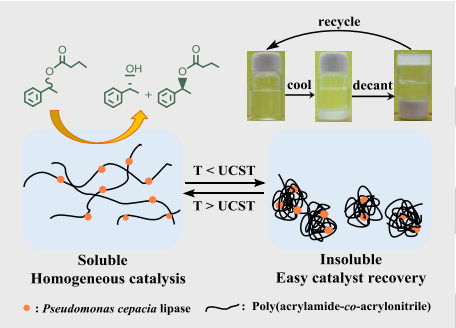


*Lan-Lan Lou, Huaxin Qu, Wenjun Yu, Bei Wang, Lezi Ouyang, Shuangxi Liu*, and Wuzong Zhou**

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**Covalently Immobilized Lipase on a Thermoresponsive Polymer with an Upper Critical Solution Temperature as an Efficient and Recyclable Asymmetric Catalyst in Aqueous Media**