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Herein, amino-epoxy supports were innovatively imported on magnetic nanoparticles for immobilizing enzymes. The acquired immobilized *Penicillium expansum* lipase with this heterofunctional support represents a novel class of heterogeneous catalyst in the synthesis of 1,3- dibutylurea from ethylene carbonate with butylamine. The yield of 1,3- dibutylurea could achieve 77% under solvent free conditions and 60°C. After completion of reaction, the catalyst was simply recovered by an external conventional magnet and recycled without significant loss in the catalytic activity (up to ten cycles).
Penicillium expansum Lipase Coated Magnetic Fe₃O₄–Polymer Hybrid Hollow Nanoparticles: Highly Recoverable and Magnetically Separable Catalyst for the Synthesis of 1,3-Dibutylurea

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Herein, amino-epoxy supports were innovatively imported on magnetic nanoparticles (Fe₃O₄-polymer hybrid nanospheres) for immobilizing enzymes. This new support has a coating layer with dual functional groups (epoxy and amino-epoxy). Thus, this support has a great anionic exchanger power and a high number of epoxy groups. The acquired immobilized Penicillium expansum lipase with this heterofunctional support represents a novel class of heterogeneous catalyst in the synthesis of 1,3-dibutylurea from ethylene carbonate with butylamine, which has not been very commonly catalyzed by enzymes. After the optimization of the reaction conditions, the yield of 1,3-dibutylurea could achieve 77% under solvent free conditions and 60°C. After completion of reaction, the catalyst was simply recovered by an external conventional magnet and recycled without significant loss in the catalytic activity (up to ten cycles).

keywords: magnetic catalysts, Lipase, Biocatalyst, chitosan, 1,3-dibutylurea

Introduction

Enzymes are versatile biocatalysts with high specificity, and their applications have been found in areas of biomaterials and organic syntheses. However, the major drawbacks for their industrial applications are low thermal stability and solvent stability as well as the difficulty in recycling. Application of lipase can be achieved more economically and efficiently by immobilization to enhance its activity, selectivity, and operation stability. For example, the activity of lipase could be improved by lipase adsorption on hydrophobic supports through the hydrophobic areas surrounding the active centre and leaving stabilized the open form of the lipase. Enantiomeric ratio may be changed from 1 to almost 100 just by using different immobilized preparations in some cases. The stability of lipase could be enhanced through rigidification of the enzyme structure by multipoint covalent immobilization. Therefore, a lot of fruitful effort has been made on the preparation of lipases in immobilized forms, which involve a variety of both support materials and immobilization methods. Nanophase materials have many advantages in immobilization due to their unique size and physical properties. Lipases, immobilized onto the nanoparticles which have high specific surface area and low diffusion resistance, possesses much higher catalytic activity and stability in spite of the cumbersome preparation, painful separation and poor efficiency. Currently, using the magnetic property is a pinpoint protocol to solve these problems. With the rapid development of nanotechnology, magnetic nanoparticles for enzymes immobilization are now being studied prosperously all over the world. However, immobilization on nanoparticles has also some drawbacks: enzymes immobilized on nanoparticles are easy to lose their activity due to the interacting with external interfaces or even enzyme molecules immobilized on other particles. This may be solved by covering the immobilized enzymes with polymers, which could provide porous structure to stabilize enzymes. Nowadays, with the flourish of green chemistry, magnetic Chitosan(CS)-Fe₃O₄ nanoparticles with their potential application for enzyme immobilization have attracted much extensive concerns because of chitosan’s excellent properties such as non-toxicity, biocompatibility, mucus-adhesion and biodegradation. Zhi et al. prepared magnetic CS-Fe₃O₄ nanoparticles in situ with tiny pools of water-in-oil microemulsion containing CS and ferrous salt as micro-reactors by adding the basic precipitant of NaOH into the micro-emulsion. The defect of this method is poor productivity; in addition, it uses a lot of surfactant and co-surfactant compounds that are pernicious to the environment. Wu et al. prepared magnetic CS-Fe₃O₄ nanoparticles by the covalent binding of CS and tripolyphosphate on the Fe₃O₄ nanoparticles by the one-step method. The problem of this method is that the magnetisation process is complicated and hard to control. Sidimiak et al. prepared CS-poly[N-benzyl-2-(methacryloyloxy)-N,N-dimethylethanaminium bromide] coated magnetic nanoparticles...
by co-precipitation method via epichlorohydrin CS cross-linking reaction. The disadvantage of this method is that it uses some poisonous compounds, such as benzyl bromide, which is nocuous and not environment benign. Ding et al.\(^{[45]}\) illustrated a successful and green synthesis route of magnetic hollow Fe\(_2\)O\(_4\)-polymer hybrid nanospheres(MHHNs) by the addition of Fe\(_2\)O\(_4\) nanoparticles to an aqueous solution of polymer-monomer pairs composed of the cationic polymer CS and the anionic monomer acrylic acid(AA), followed by polymerization of AA and selective crosslinking of CS at the end of polymerization. This innovative method, with the highlights of simple operation, high performance, cost-effective, and environment-benign, will usher in an rosy prospects for magnetic nanospheres in the application of the immobilized enzymes.

In this paper, we introduce this kind of magnetic CS-Fe\(_2\)O\(_4\) nanoparticles into immobilization of lipases. MHHNs was activated by epoxy chloropropane. After that, \textit{Penicillium expansum} lipase(PEL) was immobilized on this support by the covalent linkage between epoxy group on the supports surface and amino group on the enzymes surface to form the immobilized PEL magnetic Fe\(_2\)O\(_4\)-polymer hybrid nanospheres(MHHNs-lip). Recently, the production, purification, and application of PEL is discussed in various reviews\(^{[46,47]}\). The immobilization on this kind of supports follows the two-step mechanism\(^{[48]}\): an ionic exchange with the amino groups in the support or hydrophobic adsorption is the first step in the immobilization; In a second step, the protein which has been previously adsorbed is covalently attached to the epoxy groups present in the support surface. Finally, to finish the protein-support reaction, epoxy groups can be easily blocked by reaction with very different thiol or amine compounds under mild conditions\(^{[49]}\), preventing further uncontrolled reaction between the support and the enzyme that could decrease its stability. Obviously, the protocol is simple and allows the large-scale preparation of other enzymes, like \textit{Aspergillus niger} lipase and \textit{Rhizopus chinensis} satio lipase, which are still under investigation in our lab.

At the same time, we investigate the application of MHHNs-lip in the synthesis of 1,3-Disubstituted ureas which are very essential intermediates of pharmaceuticals and biochemicals. 1,3-Disubstituted ureas have extensive applications such as dyes, antioxidants, corrosion inhibitor, and intermediates for the preparation of pharmaceuticals and agricultural chemicals.\(^{[50,51]}\) Recently, these compounds have attracted intensive interest because substituted ureas, with amino acid groups, have been used for brain cancer treatment and have been proven to have a marked inhibitory effect on HIV protease enzyme.\(^{[52]}\) The currently studied alternative routes for the synthesis of ureas through phosgene substitutes are: (a) the oxidative carbonylation of amines with carbon monoxide under various metal catalysts.\(^{[53,54]}\) (b) the direct reaction of carbon dioxide with amines.\(^{[55,56]}\) (c) the use of some phosgene substitutes such as carbonates, that are less toxic and more stable.\(^{[57]}\) Another strategy to synthesize 1,3-disubstituted urea is the composite of amine and ethylene carbonate(ECA)(scheme 1) , while cyclic carbonates are green reagents and solvents that are industrialized obtained from an insertion reaction of carbon dioxide into epoxides.\(^{[58,59]}\) Some base catalysts such as NaOMe,\(^{[60]}\) CaO,\(^{[61]}\) 1,5,7-Triazabicyclo[4.4.0]dec-5-ene and thiourea\(^{[62]}\) or Cs\(_2\)CO\(_3\)\(^{[63]}\) were used to catalyze this reaction. However, all of these catalysts operate at extreme conditions(above 90°C) while it is complicated to recycle; Besides, most of these conventional catalysts contained metal, which are regarded as “environmental-hazardous”. Nowadays, as the universal awareness of environmental protection, eliminating metal component involvement for catalyst design and abating volatile organic solvent usage in the process of chemical synthesis is more pressing and promising for our future.

In this study, we extended the applied scope of these nanoparticles in the synthesis of 1,3-Disubstituted ureas. We report immobilization of PEL coated magnetic hollow Fe\(_2\)O\(_4\)-polymer hybrid nanospheres, which represents a novel class of heterogeneous catalyst for the synthesis of 1,3-Dibutylurea from EC with butylamine. The main superiorities behind using the immobilized PEL magnetic Fe\(_2\)O\(_4\)-polymer hybrid nanospheres(MHHNs-lip) as the catalyst were: (1) Compared to those achieved chemical catalysts, lipase is regarded as the optimized potential candidates for environmentally friendly reactions. (2) The synthesis of MHHNs-Lip is simple and allows the large-scale preparation of the nanospheres, which also follows an eco-friendly and non-toxic route. (3) The catalyst can be easily separated by an external magnet, without using extra organic solvents and additional filtration steps. (4) The chemical reaction with the catalyst of MHHNs-Lip was carried out under mild conditions (60°C and atmospheric pressure). All of them are commendably in agreement of the principles of green chemistry and sustainable development.

Results and discussion

Characterization of immobilized lipase

Typical TEM micrographs of pure Fe\(_2\)O\(_4\) and MHHNs-lip nanoparticles are shown in Fig. 1. A salient feature of Figure 1b,c,d is that these nanospheres have an intense dark circle within the shells of the spheres and dark spots at the surface of some spheres, which suggests that the Fe\(_2\)O\(_4\) particles were well coated by polymer acrylic acid(PAA). CS and PEL. The mean size of Fe\(_2\)O\(_4\) particles were 30±5nm.
The thermal stability of samples was investigated by thermogravimetric analysis (TGA). TGA curves for the particles of bare Fe$_3$O$_4$, PEL, MHHNs, and MHHNs-lip are shown in Fig. 2. As shown in Fig. 2 in all samples, weight loss within 200 °C was attributed completely to the loss of adsorbed water molecules[66]. MHHNs showed a higher weight loss than bare Fe$_3$O$_4$ because of the loss of the PAA and CS component in Fig. 2b. After immobilization of PEL, the weight loss in Fig. 2c increased again which was due to the further increased organic components on the MHHNs-lip. From this figure, the protein loading content was determined to be 56mg/g, which was very close to the calculated value(The total amount of PEL protein assayed according to the Coomassie brilliant Blue G-250 method was 58 mg/g).

Figure 3 shows the FT-IR spectra of (a) bare Fe$_3$O$_4$, (b) Penicillium expansum., (c) MHHNs-lip. The magnetic properties of the magnetic nanoparticles were analyzed by VSM at room temperature. Fig. 4 shows the hysteresis loops of the samples. The saturation magnetization is found to be 38.79 emu/g for MHHNs, respectively, less than the pure Fe$_3$O$_4$ nanoparticles (67.08 emu/g). This difference suggests that a large amount of chitosan and polymer of acrylic acid coated on the surface of Fe$_3$O$_4$ nanoparticles. The lower saturation magnetization of MHHNs-lip (25.99 emu/g) than MHHNs suggests that PEL was immobilized successfully in the MHHNs. With the large saturation magnetization, MHHNs-lip could be separated from the reaction medium rapidly and easily in a magnetic field. In addition, there is no hysteresis in the magnetization with both remanence and coercivity being zero, suggesting that these magnetic nanoparticles are superparamagnetic. When the external magnetic field is removed, the magnetic nanoparticles could be well dispersed by gentle shaking. These magnetic properties are critical in the applications of the biomedical and bioengineering fields.

The amide I band at 1605cm$^{-1}$ is mainly due to the C-O stretching vibrations, free lipase illustrates a characteristic band of amide II with the maximum of 1414cm$^{-1}$ due to N-H bending with contribution of C-N stretching vibrations. The presence of amide III band present at maximum of 1323cm$^{-1}$ is due to N-H bending with C-C and C-N stretching vibrations. The absorption band at 2938cm$^{-1}$ was due to the stretching vibrations of C-H. These significant bands were observed in the free lipase as well as MHHNs-lip (Fig. 3c) emphasizing the lipase was successfully coated onto the surface of Fe$_3$O$_4$ magnetic nanoparticles. The FT-IR spectrum of MHHNs-lip (Fig. 3c) shows that the amide I and II band of the purified acidic lipase was overlapped in 1647cm$^{-1}$ with the absorption band of the C=O groups from PAA and ammonium groups from CS. This band became weaker, which may be due to the strong hydrogen bonding between Fe$_3$O$_4$ and lipase[69]. The characteristic peak for the Fe-O group of magnetite at 598 cm$^{-1}$ in Fig. 3c was observed which indicated the successful generation of MHHNs-lip particles.

Figure 1. TEM images of the (a) bare Fe$_3$O$_4$ nanoparticles and (b,c,d) MHHNs-lip.
The preparation procedure is schematically illustrated in Scheme 2. We selected CS, which bears amino groups, as the cationic polymer and AA, which bears acid groups, as the anionic monomer. The CS and AA polymer-monomer pair and Fe\textsubscript{3}O\textsubscript{4} nanoparticles stabilized by poly(vinyl alcohol) (PVA) were mixed and formed micelles loaded with Fe\textsubscript{3}O\textsubscript{4} nanoparticles; the cores consist of the polyionic complexes of CS and AA (i.e. positively charged protonated CS chains and negatively charged dissociated AA) and the shells consist of protonated CS chains. Initiation of the polymerization of AA with potassium persulfate (K\textsubscript{2}S\textsubscript{2}O\textsubscript{8}) followed by cross-linking of the shells with glutaraldehyde (GA) at the end of polymerization led to the formation of magnetic hollow Fe\textsubscript{3}O\textsubscript{4}-polymer hybrid nanospheres\textsuperscript{[45]}.

Following the procedures described in the literature, it is hard to give the exact structure of the groups formed by the GA. However, whatever the exact structure of the GA on the support\textsuperscript{[70,71]}, the final result is a support having a fairly hydrophobic moiety formed by the GA chain and free amino groups\textsuperscript{[72]} (the ratio\textsuperscript{[73]} of aldehyde groups from GA to the amino groups from CS was 1:2). In the next activation procedure with epoxy chloropropane, the epoxy activation will proceed via this amino groups, hydroxyl\textsuperscript{[67]} and carboxyl\textsuperscript{[74]}. Therefore, the final support will be a amino-epoxy heterofunctional one(Scheme 3), which contribute a lot to promote the absorption via ionic exchange at low ionic strength\textsuperscript{[75]}. However, due to the tendency of the open form of the lipases to become adsorbed versus hydrophobic interface, even though immobilization is performed at low ionic strength, the enzyme will still be immobilized by both immobilization mechanisms: interfacial activation and ionic exchange\textsuperscript{[76-78]}. After enzyme hydrophobic adsorption, the reactive groups of the enzyme near the support surface may produce some covalent reactions, which may be produced after enzyme immobilization, and there is no guarantee that the enzyme will finally have any covalent attachment with the support\textsuperscript{[75]}. Therefore, after completion of the immobilization, in order to protein-support reaction, aspartic acid was added to block epoxy groups to prevent further uncontrolled reaction between the support and the enzyme that could decrease its stability\textsuperscript{[48,49]}.

On the basis of the two-step mechanism of enzyme immobilization\textsuperscript{[67,79]} the adsorption of the enzymes on the support surface was just the first step; In a second step, with enzymes absorbed to the surface of the support successfully, the high concentration of reactive groups in the PEL and of epoxy groups in the support could make a rapid “intramolecular” covalent reaction(Scheme 4). It has been reported that the probability of the formation of multiple covalent linkage between PEL and epoxy could be enhanced, leading to the increase in the stability PEL immobilized microspheres\textsuperscript{[67,80]}. The immobilization of enzymes inside hollow MHHNs may increase the stability by preventing any intermolecular process and by preventing the enzyme from interactions with external interfaces\textsuperscript{[81]}. Therefore, the cavities in the cellulose microspheres could provide not only a space for storage of the enzyme but also a wall shell to protect its structure and nature.
The catalytic activity of lipase from various natural sources has been directly evaluated in the transesterification of EC with butylamine. All the available lipases were screened based on their activities for the production of 1,3-dibutylurea. As shown in Table 1, PEL showed high catalytic performance for the production of 1,3-dibutylurea, while other lipases revealed little activity. Based on these results, we selected PEL as the lipase for the immobilization.

As seen in Table 2 when bare FeO<sub>3</sub> was used in the reaction medium, the yield of reaction was 6% in 24 h. Using bare MHHNs in the reaction at room temperature gave 4% yield. In another experiment using the MHHNs-1ip gave 77% yield. This value is higher than those using the same lipase in a free form, where the yields ranged from 69 to 77%. It seems that after immobilization, there was an enhancement of the enzyme activity, as shown by Dalla Vecchia in esterification reactions of carboxylic acids with n-pentanol in organic media by Rhizopus oryzae lipase. However, the activity of the immobilized lipase decreased when PEL was immobilized in conventional epoxy supports(epoxy Sepabeads). This different effect on the enzyme activity of the immobilization when using the different supports (even although having both of them epoxy groups as reactive group) may be related to different enzyme orientations of the enzyme on the support. This implies that the enzyme likely reacts with the support via different areas (the areas of the protein surface that have more hydrophobic residues or the areas of the protein that have more negative charge) with concomitant different effects on enzyme activity<sup>[48]</sup>.

The temperature influence on transesterification process has also been investigated. Seen from Table 3, the temperature increase is favourable for the 1,3-dibutylurea production, since the enzyme activity increases with the temperature increase. The yield to 1,3-Dibutylurea increased gradually with the temperature till a maximum at 60°C. However, further increase in temperature (up to 70°C) led to a slightly decrease of the yield of 1,3-Dibutylurea. Nevertheless, we adopted 60°C for this work, which is in agreement with the our earlier reports for the appropriate temperature of this lipase<sup>[43]</sup>.

**Screening of biocatalyst for 1,3-Dibutylurea synthesis**

The catalytic activity of lipase from various natural sources has been directly evaluated in the transesterification of EC with butylamine. All the available lipases were screened based on their activities for the production of 1,3-dibutylurea. As shown in Table 1, PEL showed high catalytic performance for the production of 1,3-dibutylurea, while other lipases revealed little activity. Based on these results, we selected PEL as the lipase for the immobilization.

**Table 1 Screening the lipase sources for 1,3-Dibutylurea synthesis.**

<table>
<thead>
<tr>
<th>Entry</th>
<th>Catalyst</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Penicillium expansum</td>
<td>69</td>
</tr>
<tr>
<td>2</td>
<td>Penicillium neutral expansum</td>
<td>37</td>
</tr>
<tr>
<td>3</td>
<td>Aspergillus niger</td>
<td>29</td>
</tr>
<tr>
<td>4</td>
<td>Aspergillus oryzae</td>
<td>15</td>
</tr>
<tr>
<td>5</td>
<td>Rhizopus chinensis ratio</td>
<td>10</td>
</tr>
<tr>
<td>6</td>
<td>Porcine pancreatic</td>
<td>6</td>
</tr>
<tr>
<td>7</td>
<td>Novozym 435</td>
<td>12</td>
</tr>
<tr>
<td>8</td>
<td>Neural proteolytic enzyme</td>
<td>5</td>
</tr>
<tr>
<td>9</td>
<td>Acidic proteolytic enzyme</td>
<td>3</td>
</tr>
<tr>
<td>10</td>
<td>Alkaline proteolytic enzyme</td>
<td>4</td>
</tr>
</tbody>
</table>

**Table 2 Synthesis of 1,3-Dibutylurea.**

<table>
<thead>
<tr>
<th>Entry</th>
<th>Catalyst</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PEL</td>
<td>69</td>
</tr>
<tr>
<td>2</td>
<td>FeO&lt;sub&gt;3&lt;/sub&gt;</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>MHHNs</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>MHHNs-lip</td>
<td>77</td>
</tr>
<tr>
<td>5</td>
<td>Epoxy Sepabeads-lip</td>
<td>70</td>
</tr>
</tbody>
</table>
Table 3 Influence of reaction temperature on the 1,3-dibutylurea synthesis. Conditions: MHHNs-lip catalyst: 0.7 g; EC: 40 mmol; butylamine: 80 mmol; time: 24 h.

<table>
<thead>
<tr>
<th>Temperature(℃)</th>
<th>Yield(%)</th>
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<tbody>
<tr>
<td>20</td>
<td>9</td>
</tr>
<tr>
<td>30</td>
<td>23</td>
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<tr>
<td>40</td>
<td>35</td>
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<tr>
<td>50</td>
<td>52</td>
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<tr>
<td>60</td>
<td>77</td>
</tr>
<tr>
<td>70</td>
<td>76</td>
</tr>
</tbody>
</table>

As shown in Table 4, the temperature influence on the transesterification process was also investigated. It is apparent that longer reaction time from 0 to 24 h is more advantageous for the reaction process with the improved yield. When further increasing the reaction from 24 h to 36 h, no apparent change on the 1,3-dibutylurea yield can be seen, indicating an appropriate reaction time as 24 h.

Table 4 Influence of incubation time on the 1,3-dibutylurea synthesis. Conditions: MHHNs-lip catalyst: 0.7 g; EC: 40 mmol; butylamine: 80 mmol; temperature: 60 ℃.

<table>
<thead>
<tr>
<th>incubation time(h)</th>
<th>Yield(%)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>22</td>
</tr>
<tr>
<td>3</td>
<td>34</td>
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<td>24</td>
<td>77</td>
</tr>
<tr>
<td>36</td>
<td>77</td>
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</table>

The recyclability and storage stability of lipase

To make the process more economical, it is necessary to study the recyclability of immobilized lipase, which is regarded as the most important advantage of an immobilized enzyme compared to the free enzyme. After completion of the reaction, the catalyst in free PEL catalyst reaction was recovered by centrifugation of the resulted suspension and washed using acetone. The residue obtained was dried at 45 ℃ under reduced pressure overnight (at 1 Torr for 24 h) and was then used for the next generation. At the same time, the catalyst in MHHNs-lip catalyst reaction was magnetically separated from the reaction medium directly (Fig. 6) and run in another reaction vessel under same condition. Residual activity of both catalyst during the reuse is presented in Fig. 7, which shows that MHHNs-lip has a better recyclability compared to free PEL. This suggests that the lipase immobilized on MHHNs may stand a higher stability than the original lipase. The activity of the first batch was taken to be 100%. The MHHNs-lip exhibited a better reusability, retaining 94.8% residual activity after being used 10 times. Moreover, there was no further loss in activity after these cycles. Fig. 8 shows the FT-IR spectra of MHHNs-lip before and after the catalyst was used 10 times in the reaction medium. As seen in Fig. 8, no change in FT-IR spectra of the catalyst was observed after 10 cycles. Moreover, according to elemental analysis, the organic content in the catalyst after 10 cycles was found to be C 35.5%, Fe 14.6%, and N 39.9%, and no considerable changes were seen in CHN data after recycling.

The storage stability study of free lipase and MHHNs-lip was investigated and the immobilized lipase was found to be appreciably stable for a period of 60 days retaining its original catalytic activity (Fig. 9). The immobilized enzyme provided excellent yield up to 77% of desired product whereas commercially available lipase provided 69% yield after a considerable period of 60 days.
Figure 9. The storage stability of immobilized lipase.

Figure 10. Relative activity of the free and the immobilized PEL incubated in 10mM phosphate buffer (pH 7.0) at 80°C for 12h.

**Thermal Stability of the Immobilized PEL**

The thermal stability of the immobilized PEL is one of the most important criteria dealing with its applications. Figure 10 shows the relative activity of the free and the immobilized PEL incubated in phosphate buffer at 80°C for 12h. As shown in Figure 10, the relative activity of the immobilized PEL was significantly improved through the immobilization on the MHHNs. In addition, no protein could be observed from the immobilization PEL(MHHNs-lip) even after a period of 48h under magnetic stirring, which means the mechanical stability of MHHNs-lip is also stable.

**Conclusion**

We have developed a novel class of heterogeneous biocatalyst which is particularly attractive in the practice of 1,3-dibutylurea synthesis in an environmentally friendly manner. PEL was immobilized successfully in the hollow structure of the activated MHHNs. The immobilized PEL exhibited high effective activity and thermal stability as well as good reusability. Moreover, the cellulose magnetic microspheres loaded with PEL could be conveniently and easily magnetically separated from reaction solution, leading to recovery of the catalysts. The immobilization carriers prepared from CS and acrylic acid solution in aqueous solvent via a simple, easily prepared, and environmental friendly process will have wide applications in the biocatalyst fields.

**Experimental**

**Chemical reagents**

Crude lipase from *Penicillium expansum*(PEL)(5,000Umg⁻¹ solid), *Penicillium neutral expansum*(PNEL)(10,000Umg⁻¹ solid), *Aspergillus niger*(ANL)(120,000Umg⁻¹ solid), *Rhizopus chinensis satio*(RCSL)(10,000Umg⁻¹ solid). *Aspergillus oryzae* (AOI)(30,000Umg⁻¹ solid), *Porcine pancreatic*(PPL) (135Umg⁻¹ solid), *Neural proteolytic enzyme* (50,000Umg⁻¹ solid), *Acidic proteolytic enzyme*(60,000Umg⁻¹ solid), *Alkaline proteolytic enzyme*(40,000Umg⁻¹ solid) was kindly donated by Shenzhen Leveking Bio-engineering Co. Ltd., China. These enzymes were produced by spraying the concentrated supernatant from fermentation with addition of a certain amount of starch as a thickening agent. Immobilized (Novozyme435) *C. antarctica* lipase B (EC 3.1.1.3) was donated from Novozymes (China) Investment Co. Ltd. Epoxy Sepabeads, EC, butylamine, Fe₃O₄ nanoparticles and all other chemicals were purchased from Aladdin reagent company, China and were used without further purification. Water used in the study was prepared using a Milli-Q Water Purification System (Millipore, Bedford, MA, USA).

**Procedure of preparation of magnetic hollow Fe₃O₄-polymer hybrid nanospheres**

The procedure of preparation of magnetic hollow Fe₃O₄-polymer hybrid nanospheres was carried out as reported. 10mL of a PVA-stabilized magnetite nanoparticle suspension (≈1×10⁻¹⁸ Fe₃O₄ particles mL⁻¹) was added to 50mL of an aqueous solution of CS-AA, which consisted of 0.5g of purified CS (Mw=200kDa, degree of deacetylation=90%) and 0.22g of AA, with a stoichiometric molar ratio of 1:1:1 (glucosamine unit:acid). Polymerization was then initiated by K₂S₂O₈ at 70°C under a nitrogen stream. As the reaction system appeared opalescent, the reaction was allowed to proceed for another 100minutes at 50-60°C. Afterwards, 0.5mL of GA, a bifunctional crosslinker, was added to the reaction system at 40°C to crosslink chitosan selectively. The resultant suspension was separated by an external magnet and washed using acetone. The amino bands of the shells were cross-linked with glutaraldehyde, in the ratio of glucosamine unit in CS to aldehyde unit in GA of 2. Amine moieties were quantified by measuring the color obtained by the
reaction of the primary amino groups on the support and an excess of picrylsulphonic acid solution.[84,85]. However, in order to immobilize the lipase, we need activate MHHNs with epoxy chloropropane.

**Activation of magnetic hollow Fe₃O₄-polymer hybrid nanospheres**

MHHNs(4g) were added to a solution of 12mL epoxy chloropropane in 40mL of DMSO; then, 40mL of 2 NaOH was slowly dripped in the reaction mixture system. The mixture was allowed to stand for 6 h at room temperature; then, the resulting solution was removed by magnetic separation. The activated MHHNs were obtained after washing with acetone and distilled water until the eluate was neutral.[67] The epoxyl groups in MHHNs were determined by a reaction between the oxirane ring and sodium thiosulfate, followed by titration with 0.01 mol/L hydrochloric acid to neutralize any released OH- in the reaction.[86]. The amount of epoxyl group in the activated MHHNs was estimated to be 1.68 mmol/g (dry). The epoxide group readily reacts with enzyme. Under relatively mild conditions, these groups in the cellulose support may react with both amino groups and carboxyl group in enzyme.

**Preparation of the Immobilized PEL.**

The immobilization was carried out as follows. About 1.0 g of the activated magnetic carriers was mixed with 1.0g lipase in 25mL of 10mM sodium phosphate at pH 7.0 to avoid diffusional problems that could alter the apparent properties of the immobilized enzymes under continuous and gentle stirring. The mixture was shaken at room temperature for 24 h at 250 rpm. And then, the remaining epoxy groups were blocked by incubation with 3M aspartic acid at pH 8.5 for 24 h.[87] The supernatant was removed by magnetic separation, and the MHHNs-lip was washed with deionized water, respectively, to remove the noncovalently coupled enzymes on the supports. The MHHNs-lip was stored at 4 °C. The total amount of PEL protein was assayed according to the Coomassie Brilliant Blue G-250 method with bovine serum albumin (BSA) as the standard.[88]

**Measurement of Enzyme Activity.**

*Soluble PEL Activity.* The hydrolytic activity of PEL in aqueous solution was determined according to the method described in literature [89], with a slight modification. The PEL solution (~0.23 U/ml, based on the activity of catalyzing the hydrolysis of p-nitrophenyl palmitate(pNPP) at 37°C in 50mM phosphate buffer, pH 6.0) was obtained as the supernatant after dissolution of 1.5 g of the enzyme powders in 10.0ml phosphate buffer (50mM, pH 8.0) followed by centrifugation. A substrate mixture was prepared by mixing 1ml of 15.0mM pNPP in isopropanol and 9ml of a 50mM buffer solution (phosphate buffer, pH 5.0-8.0; Tris-HCl buffer, pH 7.0-9.0; barbiturate-HCl buffer, pH 7.5-10.0) containing 0.1% (w/v) arabic gum and 0.4% (w/v) Triton X-100. A cuvette containing 2.4 ml of this substrate mixture was placed in the Pharmacia Biotech Ultraspec 2000UV/V is spectrophotometer, equipped with a thermostatic cell, for pre-equilibration at 37°C, and the reaction was started by the addition of 0.1ml of the PEL solution. The variation of the absorbance at 410nm of the assay against a blank without enzyme was monitored for 2-5min. The reaction rate was calculated from the slope of the absorbance vs. the time curve by using the molar extinction coefficient for p-nitrophenol(pNP), obtained as measured above.

*Immobilized PEL Activity.* The enzyme activity of immobilized PEL was measured in the same manner as in the determination of the soluble PEL activity described in the previous section, except that the reaction mixture was continuously stirred during the reaction. The activity yield remaining after immobilization was defined as

\[
\text{activity yield} = \frac{C}{A} \times 100 \quad (1)
\]

and the immobilization yield was calculated by

\[
\text{immobilization yield} = \frac{A - B}{A} \times 100 \quad (2)
\]

where A is the total activity of enzyme added in the initial immobilization solution, B is the activity of the residual enzyme in the immobilization and washing solution after the immobilization procedure, and C is the activity of the immobilized PEL. The maximum activity is defined as 100%, and the relative activity refers to the percentage that an enzyme activity accounts for the highest one. The concluded activity yield and immobilization yield were 55.6% and 44.8%.

**Characterization of free and immobilized lipase**

Transmission electron Microscopy(TEM) was performed with a JEM-2100FEF field emission electron microscope operating at 200kV, equipped with an EDAX Phoenix EDS analyzer. The Thermo gravimetric analysis (TGA) was carried out using Qseries 600 analyzer. About 8-10 mg of samples were placed in a ceramic crucible and the analysis was programmed from 30 to 800°C with 10°C min⁻¹ rise in temperature, under 99.99% pure nitrogen atmosphere with flow of 100mL min⁻¹. The reference run was carried out with an empty sample crucible pan and the results were recorded accordingly. The immobilized biocatalysts and free lipase were investigated for their native confirmation using FT-IR analysis (Bruker, Vertex 70). The magnetic properties of Fe₃O₄ MHHNs an MHHNs-lip were determined by a vibrating sample magnetometer(Lake Shore 7410 VSM). X-ray diffraction of the magnetic microspheres was carried out on the X-ray diffract meter (Rigaku D/MAX-2400 XRD with Ni-filtered Cu Ka radiation). The XRD patterns were recorded in the region of 20 from 10 to 80°C. Samples were ground into powders and dried in a vacuum oven at 60°C for 48 h before characterization.

**Procedure for the synthesis of 1,3-Dibutylurea from EC with butylamine**

The reaction was performed in a 25mL conical flask. After
40 mmol of EC, 80 mmol of butylamine and the lipase catalyst was charged into the reactor, the mixtures were incubated for 24 h under stirring at temperatures in the range 60°C with agitation speed of 180 rpm. At the end of the reaction, the catalyst was separated by an external magnet and washed using acetone. The residue obtained was dried at 45°C under reduced pressure overnight (at 1 Torr for 24 h) and then was used for the next generation. After separating the catalyst, the reactor was cooled to room temperature and the reaction mixture was dispersed in 50 mL of water. Then, the resulting mixture was stirred for 1 h at room temperature. The solid formed was filtered off and washed with water. The product yield was determined from the weight of the solid. The product was characterized by gas chromatography, Gas Chromatograph-Mass Spectrometer and 1H NMR; comparison with authentic samples was made whenever possible.

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Notes and references

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