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Substrate Imprinted Lipase Nanogel for One-step Synthesis of Chloramphenicol Palmitate

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Enzymatic catalysis with high enantio- and regio-selectivity, which is attractive for green synthesis of chemicals, often suffers from low activity in organic solvents utilized as reaction media. Here, we describe a 'substrate-imprinted' lipase nanogel that displays high activity in organic solvents. The first step was to encapsulate lipase into polyacrylamide nanogel by an aqueous *in situ* polymerization. Then the lipase nanogel was lyophilized in the presence of palmitic acid, a substrate of lipase, followed by the extraction with petroleum ether to remove palmitic acid from the lyophilized lipase nanogel. The imprinting treatment increased the adsorption capacity of palmitic acid by 2.9-fold and the apparent activity by 2-fold in catalyzing the transesterification reaction between para-nitrophenyl palmitate and ethanol. The effects of solvent and temperature on the yield and selectivity of the enzymatic synthesis of chloramphenicol palmitate were examined, respectively. One-step synthesis of chloramphenicol palmitate with the imprinted lipase nanogel gave a yield of ~99% and a purity of ~99% within 9 hours at 20 °C whereas the imprinted free lipase gave a yield below 60% in 20 hours. The high activity and selectivity make the substrate-imprinted enzyme nanogel an attractive catalyst for green synthesis of chemicals having complex structures.

Chloramphenicol is a bacteriostatic antimicrobial known for its broad spectrum against gram-positive and gram-negative bacteria[1,2]. Chloramphenicol palmitate has been developed as a prodrug for oral administration[3] with the advantage of quick and complete hydrolysis by intestinal esterase to release chloramphenicol throughout corporal liquids at a therapeutic level[4]. Whereas chemical synthesis of chloramphenicol palmitate is established for industrial production, the involvement of protection and deprotection steps to discriminate two available hydroxyl groups in chloramphenicol as well as the separation operations to remove impurities that have close physiochemical properties to the target compound results in an unsatisfactory yield, consumption of energy, and excess discharge of waste. Lipase is an enzyme that catalyzes the transesterification with high regio-selectivity towards hydroxyl groups, as reported elsewhere[5-7]. In fact, a number of studies have been directed to the enzymatic synthesis of chloramphenicol palmitate[8-10]. However and unfortunately, the low activity of lipase in organic media, being one to several orders of magnitude lower than that in aqueous solution, hindered the practical use of the enzymatic synthesis of chloramphenicol palmitate. The major reason that accounts for the low apparent activity of enzyme in organic solvent is that enzyme usually exists in an aggregated and rigid form in organic solvents other than the soluble and flexible one in aqueous solution. This makes the enzyme less accessible to its substrate, on one hand, and inhibits the conformational transition requested for catalysis, on the other hand. Lyophilisation with substrate is established to solve the above mentioned problem, by preserving a substrate-affinitive conformation in the active site of the enzyme[11]. Nevertheless, the apparent activities, for the most of the tested enzymes, are often far from adequate for a given application. Encapsulation of enzyme into polyacrylamide nanogel has been established as a way to enhance the thermal stability and tolerance to organic solvents[5,12-14]. In addition to strengthening the structural integrity of the encapsulated enzyme, the coverage of the hydrophilic nanogel network creates a microenvironment for enzyme to display functions as in an aqueous solution[15]. This thus comes to our mind to investigate substrate imprinted enzyme nanogel for catalysis in organic media.

Here we report our first attempt of a 'substrate-imprinted' enzyme nanogel for chemical synthesis in organic media (Figure 1). The present study started by the preparation and characterization of the substrate, palmitic acid, imprinted lipase nanogel. Then the capacity of imprinted lipase nanogel to adsorb the substrate and the activity to catalyze transesterification reactions were examined compared with non-imprinted lipase nanogel and native lipase in order to elucidate the mechanism underpinned the enzymatic catalysis. Finally the transesterification yielding chloramphenicol palmitate was

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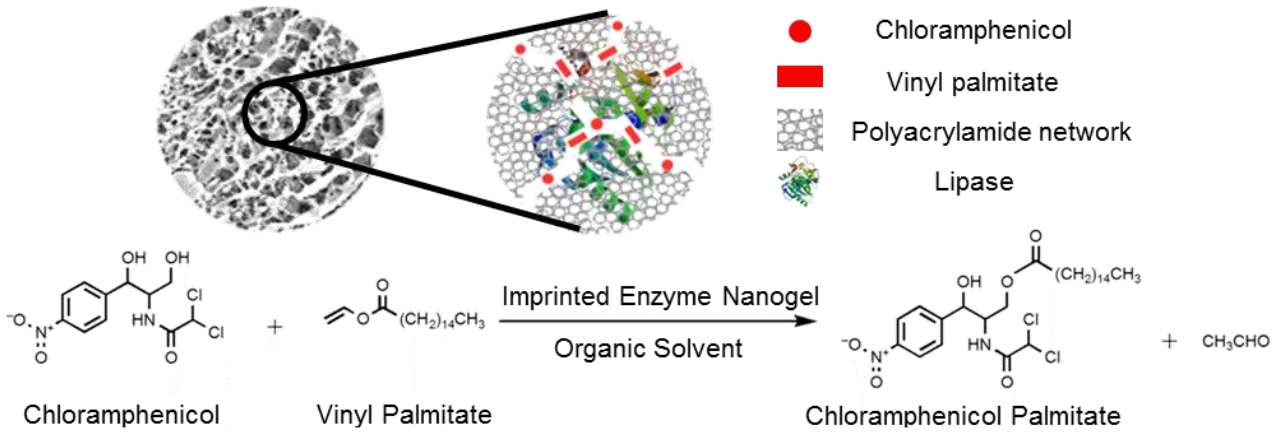

Figure 1. Enzymatic synthesis of chloramphenicol palmitate in organic solvent catalyzed by the imprinted lipase nanogel.

carried out using palmitic acid imprinted lipase nanogel to evaluate its potential for the one-step synthesis of chloramphenicol palmitate.

Lipase from *Thermomyces lanuginosus* (Lipozyme TL 100L) was encapsulated in a polyacrylamide nanogel by a two-step procedure including surface acryloylation and *in situ* aqueous polymerization[5,12-14]. First, *N*-acryloxysuccinimide (NAS) was added to the lipase solution and reacted for 6 h at pH 5.0, 30 °C, followed by the dialysis to remove unreacted reagents. Then acrylamide (AM) was added to the above lipase solution, followed by adding ammonium persulfate (APS) and *N, N, N', N'*-tetramethylethylenediamine (TEMED) as initiators. The polymerization was conducted for 12 h (See supporting information for details). The final yield of lipase nanogel is 96.7% with the hydrolytic activity of 84% compared with free lipase.

The imprinting of the lipase nanogel was carried out by adding 0.3 g of palmitic acid in 2 mL of acetone to 75 mL of acetic buffer (50 mM, pH 5.0) solution containing lipase nanogel (4 mg/mL of protein in the solution) under vigorous agitation. The mixture was then lyophilized to obtain imprinted lipase nanogel in powder form. After lyophilization, palmitic acid was extracted from the nanogel by petroleum ether, giving the substrate-imprinted lipase nanogel in powder form.

Figure 2a and 2c show the transmission electron microscope (TEM) images of negatively stained imprinted and non-imprinted lipase nanogels in aqueous solution, both of which appear spherical in shape with diameters ranging from 20 to 40 nm. Figure 2b and 2d give the scanning electron microscopy (SEM) images of the lyophilized imprinted and non-imprinted lipase nanogels in powder form. The lyophilized imprinted and non-imprinted lipase nanogels were similar in structure and insoluble in organic solvents. When carrying out catalysis in organic media, the lyophilized imprinted and non-imprinted nanogels present as aggregated powder form with porous structures shown in SEM images. The porous structure favored the uptake and access of substrate to the enzyme catalyst.

As shown by Figure 3, the imprinted lipase nanogel showed a 200% activity, compared to the native lipase in catalyzing the transesterification between para-nitrophenyl palmitate and ethanol in n-heptane (Figure 3). Imprinted native lipase gave a similar apparent activity to the non-imprinted native lipase,

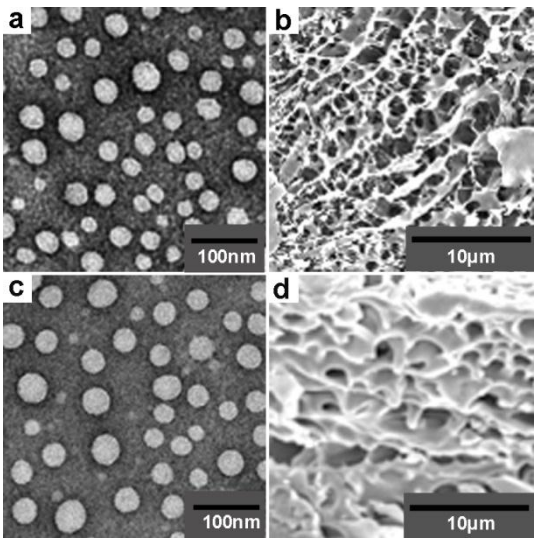

Figure 2. (a) TEM image of imprinted lipase nanogel. (b) SEM image of the powder of imprinted lipase nanogel. (c) TEM image of non-imprinted lipase nanogel. (d) SEM image of the powder of non-imprinted lipase nanogel.

suggesting that the substrate-imprinting didn't elevate the apparent activity of native lipase. Lipase nanogel without imprinting showed slightly decreased activity (~85%). This might be attributed to the additional resistance of substrate transport in the nanogel layer.

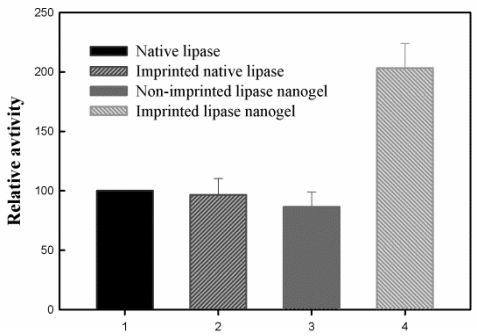

Figure 3. Relative activities of different enzyme samples in organic solvent.

To further elaborate the mechanism for the increased activity of imprinted lipase nanogel, the adsorption of the substrate vinyl palmitate by the imprinted and non-imprinted lipase nanogel was examined by incubating 50 mg of imprinted (or non-imprinted) lipase nanogel with 0.2 mg/mL of vinyl palmitate in acetonitrile at 30 °C for 7 h. As shown in Figure 4, the imprinted lipase nanogel gave a faster adsorption and a 2.9-fold increase in the adsorption capacity compared to non-imprinted counterpart (41% over 14%). The facilitated adsorption of vinyl palmitate by the imprinted lipase nanogel could be attributed to the 'imprinted affinity' of the polyacrylamide porous shell toward the substrate. This essentially facilitated the transport of the substrate from bulk solution to the encapsulated lipase. As compared with the imprinted free lipase, the 2-fold increase in the apparent activity shown by the imprinted lipase nanogel indicated that the hydrophilic environment contributed by the polyacrylamide gel network was more advantageous in providing a hydrophilic microenvironment that favours lipase catalysis.

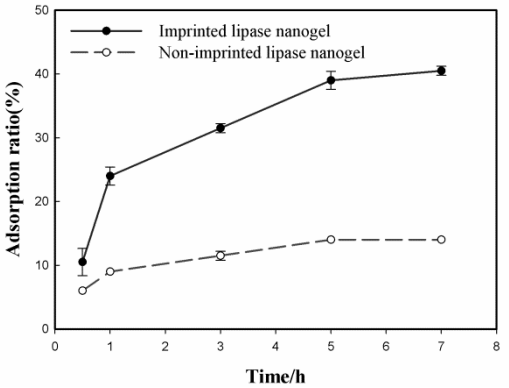

Figure 4. Adsorption of vinyl palmitate by the imprinted and non-imprinted lipase nanogel in acetonitrile.

Table 1. The purity and yield of the product at different reaction conditions.

Entry	*Solvent*	*Ratio*	*Time*	*Temperature*	*Purity*	*Yield*
1	1,4-Dioxane	1:5	48 h	30 °C	88%	10%
2	Acetonitrile	1:5	48 h	30 °C	90%	91%
3	Acetone	1:5	48 h	30 °C	72%	46%
4	Acetonitrile	1:1	48 h	30 °C	88%	69%
5	Acetonitrile	1:5	48 h	30 °C	92%	91%
6	Acetonitrile	1:10	48 h	30 °C	92%	92%
7	Acetonitrile	1:15	48 h	30 °C	90%	94%
8	Acetonitrile	1:5	48 h	20 °C	>99%	>99%
9	Acetonitrile	1:5	48 h	30 °C	90%	91%
10	Acetonitrile	1:5	48 h	40 °C	88%	81%

Chloramphenicol palmitate was synthesized using the imprinted lipase nanogel as the catalyst. The transesterification reaction between chloramphenicol and vinyl palmitate was first investigated in different solvents including 1,4-dioxane, acetone, and acetonitrile at the molar ratio of chloramphenicol to vinyl palmitate being 1:5 (Table1, Entry 1, 2, 3). For all the tested conditions, the weight percentage of the imprinted lipase nanogel in the reaction mixture was maintained at 1 wt%. After 48 h, the enzymatic process in 1,4-dioxane and acetone gave a yield of 10%, 46% and a purity of 88%, 72%, respectively. Determined by high performance liquid chromatography[16] (see supporting information), two by-products, (1R,2R)-2-[(dichloroacetyl)amino]-1-(4-nitrophenyl)-3-(palmitoyloxy)propylpalmitate [(2'R,3'R)-chloramphenicol 1',3'-dipalmitate and (1R,2R)-2-[(dichloroacetyl)amino]-3-hydroxy-1-(4-nitrophenyl)propyl decanoate (2'R,3'R)-chloramphenicol 1'-palmitate, which are the 1',3'-di-substituted product and the 1'-mono-substituted product, were formed in the reaction. When using acetonitrile as the solvent in which the reactants are soluble but the product insoluble, the yield and purity reached up to 91% and 90%.This difference in solubility facilitated the separation of product from reaction media. The molar ratio of chloramphenicol to vinyl palmitate didn't show a significant effect on the purity of product (approximately 90%) (Table1, Entry 4, 5, 6, 7). A poor yield of 69% was obtained at the molar ratio of chloramphenicol to vinyl palmitate being 1:1. When the molar ratio of chloramphenicol to vinyl palmitate was increased to 1:5, chloramphenicol was almost fully converted to chloramphenicol palmitate with a purity over 99% after 48 h reaction at 20 °C (Table 1, Entry 8). Increasing the reaction temperature up to 30 and 40 °C led to a decrease in both yield (91% and 88%) and purity (90% and 81%). This might due to the poor stability of the product and the elevated reactivity of the secondary alcohol group at higher temperature. It is also possible that the elevated temperature brings negative impact on the efficiency and selectivity of the imprinted lipase nanogel[3].

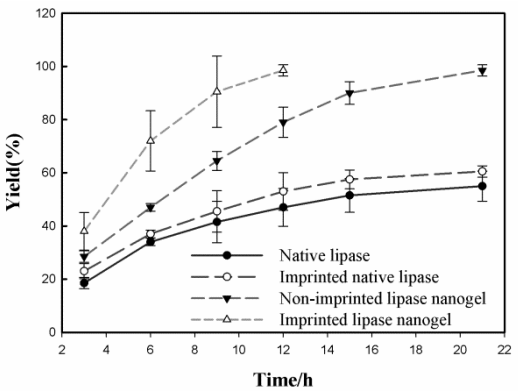

Figure 5. Conversions of chloramphenicol in the enzymatic synthesis using different preparations of the lipase catalyst.

Comparison of the transesterification reaction by different preparations of the lipase catalyst was carried out at 20 °C and a molar ratio of chloramphenicol to vinyl palmitate being 1:5. The results are shown in Figure 5. Here the imprinted lipase nanogel-catalyzed reaction gave a 99% yield of the product within 9 h. As reported by Bizerra[3], the *Candida antarctica* lipase type B (CAL-B) catalyzed synthesis of chloramphenicol palmitate takes 24 h to reach 99% yield. The non-imprinted lipase nanogel took 20 h to reach a yield of 99%. In contrast, the use of free lipase despite of imprinted or non-imprinted gave a yield below 60% in 20 h. Here again, the imprinted lipase nanogel offers the highest catalytic efficiency, which as discussed above, could be attributed to the

imprinted molecular 'cavities' in polymer shells that enhanced substrate transport and recognition, on one hand, and to the hydrophilic microenvironment of the polyacrylamide network that facilitated the conformational transition of the encapsulated lipase and thus gave a high catalytic efficiency, on the other hand. Because non-imprinted lipase nanogels do not have the affinity to substrate, it took more than 20 hours to reach ~99% yield. The low yields given by free lipase or imprinted free lipase were probably attributed to the stripping of essential water from the lipase surface by the polar solvent, acetonitrile, which led to the deactivation of the lipase[5].

Besides the transesterification reactions between chloramphenicol and vinyl palmitate, para-nitrophenyl palmitate and ethanol which were mentioned above, the imprinted lipase nanogel was also applied to catalyze the transesterification reaction between ascorbic acid and vinyl palmitate to synthesize L-ascorbyl palmitate. The results listed in table 2 show that all reactions catalyzed by the imprinted lipase nanogel have higher yields compared with those using non-imprinted lipase nanogel as the catalyst. This confirms the effectiveness of the substrate-imprinted enzyme nanogel towards different substrates.

Table 2. The yields of reactions using different substrates.

Substrate	*Nanogel*	*Solvent*	*Time*	*Temperature*	*Yield*
Chloramphenicol and vinyl palmitate	imprinted	acetonitrile	9 h	20 °C	>99%
	non-imprinted				60%
p-Nitrophenyl palmitate and ethanol	imprinted	n-heptane	1 h	40 °C	77%
	non-imprinted				66%
Ascorbic acid and vinyl palmitate	imprinted	tert-butanol	72 h	50 °C	54%
	non-imprinted				38%

In summary, we demonstrated that by using the substrate imprinted lipase nanogel, one-step synthesis of chloramphenicol palmitate with a yield of ~99% and a purity of ~99% was achieved. As the preparation of enzyme nanogel is established for enzymes having different structures[17], the substrate imprinted enzyme nanogel, with a high catalytic activity and regio- and stereo-selectivity, holds great promise for green synthesis of chemicals with complex structures in organic media[18].

Acknowledgements

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