Microreactor with mesoporous silica support layer for lipase catalyzed enantioselective transesterification†

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Lipase PS was immobilized in mesoporous silica (MPS) thin films inside a borosilicate tube microreactor for use in the enantioselective transesterification of vinyl acetate with (±)-1-phenylethanol. The immobilization was tested for 3D cubic and 2D hexagonal MPS thin films inside microreactors with and without hydrophobic treatment. The hydrophobic treatment enhanced the adsorption amount and the lipase activity for both 3D cubic and 2D hexagonal films. Of these treated films, the 3D cubic structure film exhibited the highest yield (64%) with an enantioselectivity higher than 99% in a continuous flow experiment. The activity of the immobilized lipase PS was well maintained for 36-hour continuous operation. A Ping-Pong Bi Bi kinetic model was employed to interpret the activity of the immobilized lipase PS. The ratios of the maximum velocity to the Ping-Pong constant (*i.e.*, specificity constants) were measured for lipase PS immobilized inside the microreactor and for native lipase PS. The value inside the microreactor was 800 times greater than that of the native lipase PS.

Introduction

Enzymatic reactions have acquired increasing importance as tools for fine chemical syntheses. Lipase exhibits an excellent stereoselectivity and enantioselectivity in hydrolysis and acylation reactions, and has been widely investigated over recent decades.[1–3] Lipase-catalyzed acylations are of great importance for optical resolution in chemical syntheses; however, native lipase is not uniformly dispersed in organic solvents because of its poor solubility, which results in low enzymatic activity. It is reported that the activity and enantioselectivity in the acylation of chiral alcohols catalyzed by lipase PS can be altered in various solvent systems.[4,5] Many attempts have been made to improve the lipase activity and enantioselectivity,[4–9] and a common solution is to immobilize it in support matrices, such as metal oxides, sol–gel materials, and polymers.[10–12] Lipase is uniformly dispersed in a matrix by the immobilization, and can be used effectively in organic solvent systems.

Enzyme immobilization has commonly been employed for large scale synthesis in both aqueous and organic solvents.[13] Since immobilization simplifies the separation and reuse of enzymes reducing chemical wastes, it is a valuable tool in food, pharmaceutical, and chemical industries.[14] However, by comparison with native enzymes homogeneously dissolved in an aqueous solution, the activity of enzymes in support matrices is generally reduced. This is mainly attributed to the denaturation of the enzymes and to the diffusion limitation of the reactants. As regards diffusion limitation in particular, if enzymes are immobilized deep inside a matrix, the probability of the reactant molecules encountering enzymes is limited because of the slow molecular diffusion inside the support matrix. It is inferred that enzymes immobilized near the matrix surface are mainly exploited in the reactions. Therefore, if the diffusion limitation in the support matrix is diminished, the apparent lipase activity would be enhanced.

A microreactor with a small reaction space in the range from 100 μm to 1 mm is a useful tool for fine chemical synthesis.[15–17] One characteristic feature is that it has a large interfacial area per unit volume. If a catalyst is coated on its wall, reactant molecules can easily encounter the catalyst because of the short diffusion distance inside the microreactor. Such a system can be expected to be an efficient tool for heterogeneous catalytic reactions.[18] Microreactors have increasingly been employed for enzymatic reactions as high throughput analytical and synthetic tools.[14,19] In these applications, enzymes were immobilized inside microreactors with elegant techniques including biotin–avidin labeling techniques,[20–22] cross-linking enzyme aggregation,[23,24] and use of metal oxide monolith/beads.[25–28] A simple and robust enzyme immobilization method is desired for further use of microreactors.

Recently, we have developed a microreactor that has a mesoporous silica (MPS) thin film on its inner walls.[29,30] MPS, which is typically synthesized with a silica precursor and surfactant micelle templates, and possesses a highly ordered pore structure.[31–34] Because of its uniform pores (about 7.5 nm in diameter), MPS is suitable for use as a catalyst support, and has recently been used as an enzyme support matrix.[35] Several types of MPS powder exhibited high enzyme loading capacities for horseradish peroxidase,[36] catalase,[37] and lipase.[38–43] It is also reported that this immobilization technique often enhanced the thermal and chemical stabilities of enzymes.[36,37]

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Therefore, the MPS film was provided inside the microreactor as a support matrix. Importantly, the thin MPS film (less than 150 nm) could minimize the diffusion limitation within the MPS support matrix, and enhance the apparent activity of immobilized enzymes. In this context, a microreactor containing MPS films is better adapted to enzymatic reactions in fine chemical syntheses.

This study describes the immobilization of lipase PS in MPS thin films inside a microreactor for use in the acylation of (±)-1-phenylethanol with vinyl acetate. This paper has two main parts. The first part describes the testing of several types of MPS film appropriate for lipase immobilization, and the second part evaluates the immobilized enzyme activity by comparison with native lipase PS. The surface hydrophilicity and porous structure of MPS films are discussed in the first part with a view to achieving effective lipase immobilization. It is known that the hydrophilicity of SiO_2 considerably alters the adsorption property of lipase.[12,44] The porous structure of MPS film affects the access of reactant molecules to the immobilized lipase. These two factors would play an important role in lipase activity. In the second part, the activity of the immobilized lipase is quantified by fitting to a Ping-Pong Bi Bi kinetic model for comparison with native lipase.[13]

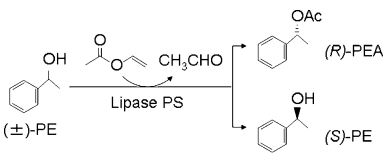
Experimental

All chemicals were used as received. For the MPS preparation, ethanol, tetraethyl orthosilicate (TEOS), and hydrochloric acid were obtained from Junsei Chemical Co. (Tokyo, Japan). F127 block copolymer supplied by BASF (Mount Olive, NJ, USA) was used as a structure-directing agent. A borosilicate tube (Vitrocom, Mountain Lakes, NJ, USA) containing six bores 0.20 mm in inner diameter and 30 cm long (3 mm outer diameter) was used as a microreactor. Lipase PS SD and lipase PS IM are products of Amano Pharmaceutical Co. Note that lipase PS IM is a commercial formulation of lipase PS immobilized on SiO_2 (diatomaceous earth). For a buffer solution, 2-(*N*-morpholino)ethanesulfonic acid (MES) was obtained from Dojin Chemical (Kumamoto, Japan). For the transesterification, (±)-1-phenylethanol (PE) and (*S*)-(–)-1-phenylethanol ((*S*)-PE) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan); (±)-1-phenylethylacetate (PEA) was purchased from Merck Schuchardt OHG (Hohenbrunn, Germany); vinyl acetate (VA) and diisopropyl ether (iPr$_2$O) were obtained from Kishida Chemical Co. (Osaka, Japan); 1,1,1,3,3,3-hexamethyldisilazane (HMDS) was purchased from Aldrich (Milwaukee, WI, USA).

The preparation of MPS thin films inside microreactors has been detailed elsewhere.[29,30] Two different MPS structures were formed in the present study. The molar ratios of TEOS, H_2O, HCl, EtOH, and F127 in precursor solutions were 1 : 9.2 : 0.042 : 51 : 0.0041 for 3D cubic MPS film and 1 : 9.2 : 0.021 : 40 : 0.0072 for 2D hexagonal film. The inner walls of micro-capillary tubes were coated with these precursor solutions. In fact, it is difficult to measure the amount of adsorption inside microreactors directly. Accordingly, a quartz microbalance system (QCM: Q-Sense D300, Göteborg, Sweden) was used for measuring the amount of lipase adsorbed on the MPS films. The same precursor solutions were used to form MPS thin films on QCM oscillators by dip coating with a 3.6 cm min^{-1} withdrawing speed. The hydrophobic surfaces were prepared with HMDS. The prepared MPS thin films were exposed to HMDS vapor at room temperature for 2 h. After this exposure, the films were dried at 70 °C to remove excess adsorbed HMDS. In this process, the surface Si–OH groups of MPS were converted into methyl terminals and became hydrophobic.[45] Surface and cross-sectional images of the prepared MPS films were acquired with a scanning electron microscope (SEM) at an acceleration voltage of 0.8–1.5 kV (S-4800 FE-SEM: Hitachi High-Technologies, Japan). For this observation, the micro-capillary tubes were broken to reveal the MPS films coated on their inner walls.

In the lipase immobilization process, the developed microreactor was filled with a 10 mg mL^{-1} lipase PS solution and kept at 5 °C for 12 h to reach adsorption equilibrium. The lipase solution was prepared by dissolving lipase PS SD in 20 mM MES buffer solution controlled at pH 7. Prior to the experiment, the microreactor was rinsed with the buffer solution for more than 30 min to remove excess lipase. Subsequently, N_2 gas was fed through the microreactor to remove any remaining buffer solution because the reactant solution is not soluble in water. In the QCM analyses used to measure the amount of adsorbed lipase, 10 mg mL^{-1} lipase solution was fed into the system where it remained for 2 h. After flowing the buffer solution to remove excess lipase near the surface, the mass change of the QCM oscillator was monitored.

Transesterification was carried out with lipase immobilized inside the microreactor. All the experiments using the microreactor were performed at 40 °C in a continuous flow manner. The reaction is expressed as follows:This model reaction catalyzed



by lipase PS is known as a route to enantiomerically pure compounds under mild conditions.[3] Because of its high activity and selectivity, the reaction is frequently discussed in the field of green chemistry.[4,46] A mixture of (±)-PE and VA dissolved in iPr$_2$O was fed at a rate ranging from 0.3 to 24 μL min^{-1} by using a syringe pump (PHD2000, Harvard Apparatus, Holliston, MA, USA). The initial concentration was ranging from 5 to 26 mM for PE and from 5 to 50 mM for VA, respectively. The effluent was collected to measure its concentration with an HPLC (Shimadzu LC–10A, Kyoto, Japan) equipped with a Chiralcel OD column (Daicel Chemical Industries, Tokyo, Japan). The UV detector was set at 254 nm, and the column temperature was held at 30 °C. The mobile phase was hexane/2-propanol (6/1), and the flow rate was 1 mL min^{-1}. Reference compounds including (±)-1-phenylethanol, (*S*)-(–)-1-phenylethanol, and (±)-1-phenylethylacetate were used to identify the retention time of the products.

Additional batch experiments were conducted using lipase PS SD and lipase PS IM to evaluate enzyme activity. The mixture of PE and VA dissolved in iPr$_2$O (3 mL) was added to a 13.5 mL glass vial containing lipase PS SD (30 mg) or lipase PS IM (5 mg), and was then stirred (600 rpm) at 40 °C. The initial concentration

of PE tested in these batch experiments was ranging from 6 to 26 mM; the initial concentration of VA was ranging from 5.7 to 57 mM. After completion of the reaction time, the suspension was quickly filtered through a cellulose acetate filter (0.5 μm, Advantec DISMIC-13, Tokyo, Japan) and then was injected into the HPLC system.

Results and discussion

MPS films for lipase PS immobilization

The first part of this study focuses on the influence of the porous structure and hydrophilicity of MPS thin films on the amount and activity of adsorbed lipase. Fig. 1 shows SEM images of MPS thin films that were formed on the inner wall of the microreactor. Two forms of MPS were prepared for the investigation: 3D-cubic (Fig. 1a and b) and 2D-hexagonal (Fig. 1c and d). A major difference between two structures was the existence of open mesopores on the surface. Uniform open pores about 7.5 nm in diameter are aligned in an orderly way with 3-fold symmetry on the surface (Fig. 1a). This is a typical feature of a 3D cubic structure.[30,34] As shown in Fig. 1b, the film thickness is about 45 nm. With the 2D hexagonal film shown in Fig. 1c, the pore channels are aligned parallel to the surface forming a swirling pattern with few open pores (dark spots in the figure) on the surface. Fig. 1d shows that the film thickness is about 125 nm and that the pore channel size is about 7.5 nm, which is almost the same as the pore size of 3D cubic film. We confirmed that ordered pore structures were present inside the microreactor. We also confirmed that the films on the QCM oscillators had the same structures as those inside the microreactor.

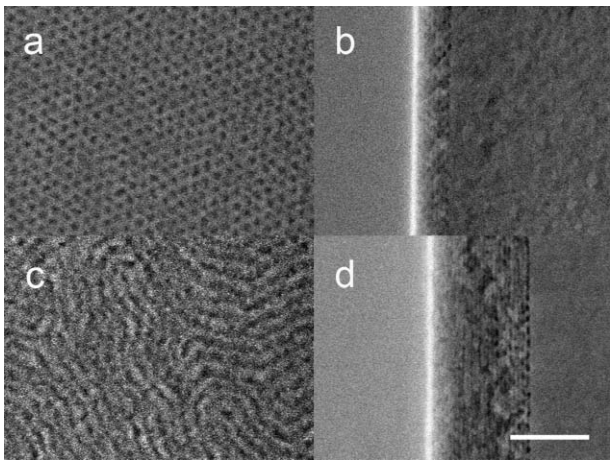


Fig. 1 Structures of mesoporous thin films on the inner wall of microreactors: (a) Surface image of 3D cubic; (b) cross-sectional image of 3D cubic; (c) surface image of 2D hexagonal, (d) cross section of 2D hexagonal. Scale bar: 100 nm.

The amount of lipase adsorbed on the MPS films on QCM oscillators was measured in order to understand the properties of support matrices suitable for lipase immobilization. We investigated MPS films with and without HMDS hydrophobic treatment. Hereafter, we refer to HMDS-treated 3D cubic and 2D hexagonal films as H-cubic and H-hexagonal films, respectively. The mass change (ΔM) of QCM oscillators coated with MPS films was monitored after they were exposed to lipase solution (Fig. 2). Fig. 2 shows that lipase adsorption increased rapidly and reached equilibrium after exposure to lipase solution. Importantly, ΔM remained constant after it was rinsed with buffer solution, which indicates that little lipase leached from the MPS films. Although several reports mention that a strong interaction between enzyme and silica is required in the immobilization process to minimize the amount of enzyme leaching out from silica,[40,47] the MPS films exhibited excellent affinity to lipase PS. The final values of ΔM are also listed in Table 1. A comparison of the final ΔM values for MPS films showed that lipase adsorbed more on HMDS-treated films than on untreated films. The lipase adsorption on H-cubic film (1.55 μg cm^{-2}) was about 9 times that on the untreated film (0.173 μg cm^{-2}), while the adsorption on H-hexagonal film (2.12 μg cm^{-2}) was about 20 times that on the untreated film

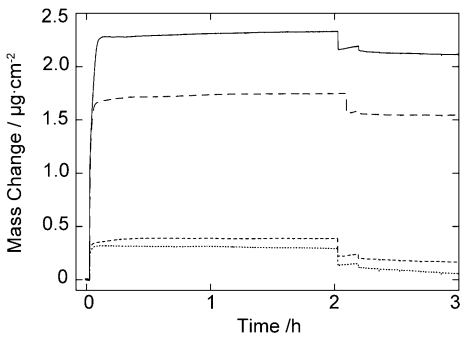


Fig. 2 Amount of lipase adsorbed in MPS films on QCM oscillators: (from top to bottom) solid line: H-hexagonal, long-dashed line: H-hubic, short-dashed line: cubic, dotted line: hexagonal. At 2 min, lipase solution was supplied to the QCM system. Every 10 min after 122 min, the buffer solution was supplied to remove excess lipase.

Table 1 Amount and activity of lipase adsorbed on 4 types of MPS thin film

Type of MPS	ΔM/μg cm^{-2}[a]	(*R*)-PEA/mM[b]	(*R*)-PE/mM[b]	(*S*)-PE/mM[b]	i-(*R*)-PE/mM[c]	i-(*S*)-PE/mM[c]	Yield [%][d]
Cubic	0.173	0.272	12.9	12.9	13.0	12.9	2.10
Hexagonal	0.102	0.202	13.1	12.4	13.0	12.9	1.56
H-Cubic	1.55	9.53	5.44	14.9	14.9	14.9	63.9
H-Hexagonal	2.12	6.64	8.31	14.8	14.9	14.9	44.7
H-Cubic[e]	—	11.2	1.17	12.5	12.1	12.1	92.5

[a] Mass change (ΔM) was calculated from frequency shift (Δf) of QCM based on Sauerbrey equation. [b] The concentration in the effluent. [c] The concentration in the influent. [d] Ratio of the concentration of (*R*)-PEA in the effluent and the initial concentration of (*R*)-PE. [e] Microreactor experiment was at a flow rate of 0.3 μL min^{-1}. Other experiments were at 1.2 μL min^{-1}. When the flow rate is small in this system, the reaction proceeds further because of long contacting time.

(0.102 μg cm^{-2}). It is clear that lipase was preferentially adsorbed on the hydrophobic films. This agrees with other reports on silica and MPS.[12,39,44] While several types of interaction including electrostatics, hydrogen bonding, hydrophobic interactions, and van der Waals interactions govern protein adsorptions,[48] the hydrophobic interaction dominates in the lipase adsorption in MPS. Furthermore, the QCM results showed that more lipase was adsorbed on H-hexagonal film than on H-cubic film. It is simply because the thickness of H-hexagonal film is greater than that of H-cubic film. As shown in Fig. 1b and d, the hexagonal film (125 nm) was thicker than the cubic film (45 nm). Because thicker films should have a larger pore volume, the H-hexagonal film had a greater lipase adsorption capacity than the H-cubic film. From the ΔM values and the size of lipase, we calculated the total volume of adsorbed lipase and compared it with the size of the pore volume. From this estimation, lipase was adsorbed on almost the entire H-cubic film from top to bottom, while an open space still remained in the H-hexagonal film. Therefore, lipase was adsorbed more on the H-hexagonal film because it even migrated inside the narrow pore channels of the H-hexagonal film during the long immobilization process.

The activity of the adsorbed lipase was also compared with four types of MPS film coated inside the microreactors. Transesterification was carried out in a continuous flow manner at a rate of 1.2 μL min^{-1}.[49] The measured concentration of the effluent flow is summarized in Table 1. The product concentrations, (*R*)-PEA, for the HMDS-treated films (9.53 mM for H-cubic and 6.64 mM for H-hexagonal) were about 35 times greater than that for the bare films (0.272 mM for cubic and 0.202 mM for hexagonal). This supports the QCM results showing that more lipase was adsorbed on the hydrophobic surface than on the hydrophilic surface. The result also suggests that lipase immobilized on the hydrophobic surface maintained good enzymatic activity and that denaturation *via* the adsorption process was not significant in the current system. Furthermore, it should be noted that while H-cubic film had only 9 times more lipase adsorption than the untreated cubic film, it exhibited a 35-fold increment in the product concentration. In other words, lipase adsorbed on H-cubic film was more active than that on the untreated cubic film. It is reported that the lipase active site is covered by a lid domain and that the lid moves away when lipase is exposed to hydrophobic environments.[50] An open form of lipase is thought to exhibit good activity. The hydrophobic treatment in the present study also had a similar effect on the transesterification catalyzed by lipase. Indeed, both H-cubic and H-hexagonal films had similar acceleration effects on the reaction. The results indicate that the hydrophobic treatment of MPS thin film enhanced the catalytic activity of immobilized lipase as well as the amount of adsorption.

We discuss the effect of MPS structure on the activity of immobilized lipase by comparing H-cubic and H-hexagonal films. As shown in Table 1, the (*R*)-PEA concentration of the H-hexagonal film (6.64 mM) was 30% less than that of the H-cubic film (9.53 mM), whereas the lipase adsorption of the H-hexagonal film (2.12 μg cm^{-2}) was 70% more than that of the H-cubic film (1.55 μg cm^{-2}). Interestingly, the reaction rate of lipase immobilized in the H-hexagonal film was slower than that in the H-cubic film. We speculate that the slow reaction rate was due to reactant molecules' access to the lipase inside H-hexagonal film. The H-hexagonal film has a limited number of open pores on the surface and appears to provide poor accessibility to its pores through its surface compared with H-cubic film (see Fig. 1c).[30,34] In some cases, there are micropores (smaller than 2 nm) on the walls of mesopore channels. However, the diffusion of reactant molecules through such micropores is presumably small compared with the diffusion through the open mesopores in Fig. 1a. Unlike the H-hexagonal film, the H-cubic film has many open mesopores on its surface, and the reactant molecules have good accessibility to the lipase immobilized inside the mesopores. Since the reaction was conducted in a continuous flow manner, its reaction rate was greatly affected by diffusion limitation. Hence, the reaction rate of immobilized lipase is correlated with the MPS film structure in terms of reactant molecules' accessibility to lipase inside the pores. Of the MPS films tested in this study, the H-cubic film is the most suitable for transesterification with immobilized lipase.

In our preliminary experiments, lipase PS SD and lipase PS IM showed good enantioselectivity in batch reactions. However, it should be noted that the enantioselectivity of lipase PS is dependent on its solvent environment and is sometimes altered by the addition of ionic liquids.[1,4] In this context, the enantioselectivity in the experiments was judged from the (*S*)-PEA and (*S*)-PE concentrations in Table 1. In fact, the concentration of (*S*)-PEA was lower than the detection limit; the change in the (*S*)-PE concentration was negligible. Hence, in all cases, the enantiomeric excess values were higher than 99%. For the H-cubic film, the enantioselectivity was high even at a yield of 92.5% in an additional experiment with a flow rate of 0.3 μL min^{-1}. Thus, the lipase immobilized in the MPS films maintains good enantioselectivity.

Evaluation of immobilized lipase PS

The H-cubic film exhibited a good capacity for lipase immobilization based on an activity test for four types of MPS film. The second part of this study focuses on the characteristics of lipase immobilized in the H-cubic film. The reactant solution was continuously fed into a microreactor containing lipase PS at a rate of 3 μL min^{-1} for 36 h, and the product concentration in the effluent solution was monitored to determine its stability (Fig. 3). The concentration after the first hour was 6.21 mM, which corresponds to a yield of 51% in an additional experiment with a flow rate of

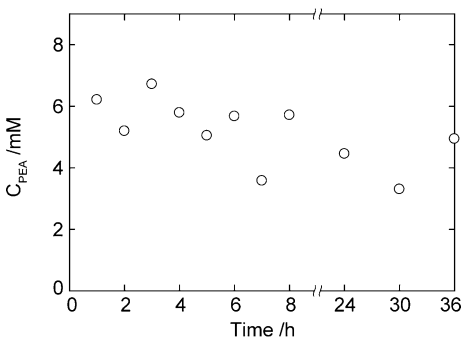


Fig. 3 Changes in the product concentration in the effluent solution with time in long-term continuous test. The reactant solution ($C_{(R)\text{-PE}}$ = 12.1 mM, $C_{(S)\text{-PE}}$ = 12.1 mM, C_{VA} = 50 mM) was continuously fed at 3 μL min^{-1} over 36 h. The temperature of the reactor was kept at 40 °C.

concentration at 8 h was 5.71 mM and the final concentration at 36 h was 4.94 mM, which was about 80% of the original value.[51] The results indicate that the reaction rate slowly decreased with time. Since a small amount of lipase was leached out in the QCM experiments, the decrease in the reaction rate was largely due to the natural decay of lipase activity. It is well known that lipase loses its activity in organic solvents.[41,52] Amano Pharmaceutical Co. also reported that lipase PS IM loses about 20% of its activity after being stirred in iPr$_2$O at 25 °C for 24 h. Therefore, the lipase immobilized in the H-cubic film provided fairly good stability, based on the result showing that it maintained about 80% of its original activity after 36 h of continuous reaction at 40 °C.

The enzymatic activity of lipase immobilized in MPS films was quantified for evaluation through a comparison with native lipase and a commercial immobilized lipase PS. Since the MPS film coated inside the microreactor was designed for continuous operation, we conducted tests with various reactant concentrations at a flow rate of 24 μL min^{-1}, and calculated the reaction rates from the product concentrations. Compared with the flow rate in the previously described activity test (1.2 μL min^{-1}), the rate in this experiment is fast (24 μL min^{-1}). Under this condition, the change in the reactant concentration is very small because of the short contact time. This allows us to assume that the reactant concentration is constant across the microreactor and then to calculate the reaction rates directly from product concentrations and flow rates.[29] It should be noted that the rates obtained with this method are equivalent to the initial rates in the batch experiments.

Fig. 4a shows the reaction rates as a function of VA concentration at (*R*)-PE concentrations, $C_{(R)\text{-PE}}$ of 2.5, 5, 6, and 13 mM. The reaction rates gradually increase with VA concentration and reach a plateau at high VA concentrations. This feature is observed for all PE concentrations. For further analysis, the results were re-plotted in Lineweaver–Burk form, where the reciprocals of the reaction rates were plotted against the reciprocal of the VA concentration, C_{VA} (Fig. 4b). Data sets at a constant $C_{(R)\text{-PE}}$ form straight parallel lines. We also confirmed that the y-intercepts of the straight lines are proportional to the reciprocal of $C_{(R)\text{-PE}}$. These are typical features representing the Ping-Pong Bi Bi mechanism.[13] In this transesterification, VA first binds to lipase to give an intermediate form that then reacts with PE to yield the product, PEA. It is known that PE sometimes acts as a competitive inhibitor in the first step of this reaction.[53–55] The current results show that the rate increased with the PE concentration (Fig. 4a), and suggest that PE inhibition was minimal under these conditions. This is because we set the initial reactant concentrations at relatively low values in order to quantify the initial activity of lipase immobilized in MPS films in the absence of competitive inhibition. While many kinetic forms have been proposed for the interpretation of transesterification,[56–58] the Ping-Pong Bi Bi mechanism in the absence of competitive inhibition was suitable for acquiring kinetic parameters in this study and is expressed as follow:

$$r_{(C_{\text{VA}},C_{(R)\text{-PE}})} = \frac{V_{\max} C_{\text{VA}} C_{(R)\text{-PE}}}{C_{\text{VA}} C_{(R)\text{-PE}} + K_{\text{PE}} C_{\text{VA}} + K_{\text{VA}} C_{(R)\text{-PE}}} \quad (1)$$

where $V_{\max}$ is the maximum velocity (mol s^{-1} g$_{\text{lipase}}^{-1}$); K_{VA} and K_{PE} are the Ping-Pong constants for VA and PE (M). Since the

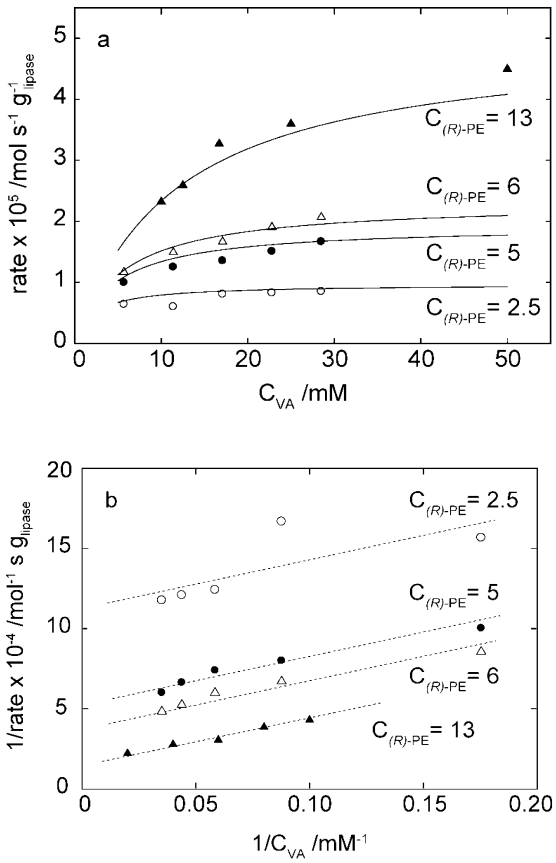


Fig. 4 (a) Transesterification experiments as a function of VA concentration. The solid lines are the fitted results based on the Ping-Pong Bi Bi mechanism. In the graph, $C_{(R)\text{-PE}}$ is the concentration of (*R*)-PE in mM. ▲: $C_{(R)\text{-PE}} = 13$ mM, Δ: $C_{(R)\text{-PE}} = 6$ mM, ●: $C_{(R)\text{-PE}} = 5$ mM, ◇: $C_{(R)\text{-PE}} = 2.5$ mM. (b) Lineweaver–Burk plot of the transesterification experiments in Fig. 4a. Dotted lines are guides to the eye.

substrate concentrations (C_{VA} and $C_{(R)\text{-PE}}$) were set at very low values, eqn (1) can be simplified as follows:[54,55]

$$r_{(C_{\text{VA}},C_{(R)\text{-PE}})} = \frac{1}{\frac{K_{\text{VA}}}{V_{\max} C_{\text{VA}}} + \frac{K_{\text{PE}}}{V_{\max} C_{(R)\text{-PE}}}} \quad (2)$$

In eqn (2), it is estimated that $1/V_{\max}$ is far smaller than $K_{\text{VA}}/V_{\max}C_{\text{VA}}$ and $K_{\text{PE}}/V_{\max}C_{(R)\text{-PE}}$. The specificity constants, which correspond to $V_{\max}/K_{\text{VA}}$ and $V_{\max}/K_{\text{PE}}$, indicate the efficiency of an enzyme that converts a substrate into a product. Hence, instead of estimating the individual parameters of $V_{\max}$, K_{PE}, and K_{VA}, the specificity constants were obtained by fitting the results to eqn (2) to evaluate the immobilized lipase activity. In addition, the slope of the straight lines in the Lineweaver–Burk plot corresponds to $K_{\text{VA}}/V_{\max}$, which is the reciprocal of the specificity constant (Fig. 4b). To estimate commercial lipase activity, the initial reaction rates were measured in batch experiments using lipase PS SD and lipase PS IM. The results of the batch experiments were also fitted to eqn (2) in the same manner. The obtained specificity constants of immobilized lipase, lipase PS SD, and lipase PS IM are summarized in Table 2.

The values of $V_{\max}/K_{\text{VA}}$ and $V_{\max}/K_{\text{PE}}$ for lipase immobilized in the MPS film are roughly the same, which indicates that the Ping-Pong constants, K_{VA} and K_{PE} are not very different

Table 2 Specificity constants of lipase PS

Type of lipase	V_{max}/K_{VA} [L s^{-1} g_{lipase}^{-1}]	V_{max}/K_{PE} [L s^{-1} g_{lipase}^{-1}]
Immobilized	4.50×10^{-3}	3.86×10^{-3}
Lipase PS SD	5.71×10^{-6}	6.85×10^{-6}
Lipase PS IM	1.17×10^{-4}	3.08×10^{-4}

from each other. The specificity constant V_{max}/K_{VA} for lipase immobilized in the MPS film is greater than that for native lipase PS SD by a factor of nearly 800. As described in the introduction, lipase PS was not dissolved in organic solvents and was not equally dispersed in the solution. This low solubility caused the native lipase activity to be low in organic solutions. Hence, the results suggest that the lipase immobilized in the MPS film encountered reactant molecules with an increased frequency, which led to excellent enzymatic activity in the transesterification. The specificity constant of lipase PS IM in Table 2 was estimated for the total weight which includes diatomaceous earth and lipase because the immobilization percentage was not provided. Although the specificity constant of lipase PS IM is not directly comparable, the specificity constant for the lipase immobilized in the MPS film (4.50×10^{-3} L s^{-1} g^{-1}) is about 40 times that for lipase PS IM (1.17×10^{-4} L s^{-1} g^{-1}). We also estimated the amount of lipase immobilized in lipase PS IM by measuring the protein concentrations of lipase PS IM and lipase PS SD using standard BCA protein assays (Pierce BCA Protein Assay Kit, Rockford, IL, USA). The estimated immobilization percentage of lipase PS IM was 34%. If this immobilization percentage (34%) is included in the values for lipase PS IM, the specificity constants were 3.44×10^{-4} L s^{-1} g^{-1} for V_{max}/K_{VA} and 9.06×10^{-4} L s^{-1} g^{-1} for V_{max}/K_{PE}. The V_{max}/K_{VA} value of lipase PS IM is smaller than that for lipase PS immobilized in the MPS films by a factor of 13. Hence, the activity of lipase PS immobilized in the MPS film was much greater than that of commercial immobilized lipase PS.

Conclusion

Microreactors with MPS thin films were employed for enantioselective transesterification catalyzed by immobilized lipase. The porous structure and surface hydrophilicity of the MPS film affected the adsorption amount and the activity of lipase PS. Hydrophobic treatment enhanced both the adsorption amount of lipase PS in the MPS films and its enzymatic activity. MPS film with open pores on the surface, which possessed good reactant accessibility, exhibited an increased reaction rate. Of the films tested in this study, lipase PS immobilized in the 3D cubic film with hydrophobic treatment exhibited the highest activity while maintaining enantioselectivity higher than 99%. The immobilized lipase also showed good stability during 36-hour continuous operation. The activity of lipase immobilized in the MPS film was quantified by fitting the results to the Ping-Pong Bi Bi mechanism. The specificity constant of immobilized lipase was 800 times greater than that of native lipase PS and was 13 times greater than that of lipase PS IM. The immobilized lipase PS in the MPS film inside the microreactor exhibited the enzymatic activity higher than the native lipase PS and lipase PS IM. A microreactor containing MPS film showed good applicability to enantioselective chemical reactions catalyzed by immobilized enzymes.

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