Architecture and Performance of Mesoporous Silica-Lipase Hybrids via Non-Covalent Interfacial Adsorption

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DOI 10.1002/aic.11963
Published online August 11, 2009 in Wiley InterScience (www.interscience.wiley.com).

To investigate the effects of surface property of mesoporous supports on the lipase immobilization and the performance of immobilized lipase, the mesoporous molecular sieve SBA-15 is functionalized with three organic moieties, dimethyl (DM), diisopropyl (DIP), and diisobutyl (DIB), respectively, by post-synthesis grafting and one-pot synthesis methods. Porcine pancreas lipase (PPL) is immobilized on SBA-15 supports through hydrogen bonding and hydrophobic interaction. The hydrophobic adsorption involves no active sites of PPL, and neither hyper-activation nor total inactivation occurs. The study on the intrinsic stability of PPL, including thermal stability, pH stability, and storage stability, indicates that the entrapment in mesoporous supports, and especially in organic-functionalized supports, makes PPL more resistant to temperature increment but more sensitive to pH change. The reusability investigation shows that the organic modification of mesoporous surface inhibits the enzyme leaching to some extent, resulting in a better operational stability. © 2009 American Institute of Chemical Engineers *AIChE J,* 56: 506–514, 2010

Keywords: porcine pancreatic lipase, SBA-15, hydrophobicity, interfacial adsorption, enzyme immobilization

Introduction

Lipase (EC 3.1.1.3) is defined as a carboxylesterase, which catalyses the hydrolysis and synthesis of long-chain acylglycerols with trioleoylglycerol being the standard substrate.[1] In fact, lipases not only hydrolyze fat or interesterify triglycerides, but are also able to catalyze the acylation or deacylation of a wide range of unnatural substrates. Because of their significant application in both hydrolysis and synthesis reactions, lipases have become of great interest to the chemical and pharmaceutical industries in recent years. To design robust biocatalysts and optimize their performance, many bio-related hybrids have been developed using organic polymers,[2,3] inorganic materials,[4,5] or hybrid composites[6] as supports through various immobilization methods including adsorption, entrapment, covalent bonding, and cross-linking. Review articles on immobilized lipases are available elsewhere.[7–9] The immobilized lipases display a number of advantages over the soluble ones, such as the possibility of recovery and recycle, simplicity in operation, and probably improvement of activity or stability. Though a lot of studies on these bioactive hybrids have been carried out, the nature of the interaction between lipase and support is still difficult to clearly elucidate. Moreover, there is not a universal rule for all the support-lipase hybrids. Immobilization does not necessarily lead to stabilization and lipase might become robust or sensitive to the environment. Lipase immobilization, far from an already solved problem, thus constitutes an exciting research field in both laboratory scale and industrial production. Recently, mesoporous silica materials have received much attention as promising supports for enzyme immobilization due to their well-ordered structures, nano-

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sized channels, and large surface areas.[4,10–12] What's more, their tunable pore sizes and surface properties make it possible to study the effects of supports on the microenvironments of enzymes.

In our previous work, we have architected a bioreactor by reducing the pore opening of SBA-15 with lipase entrapped to prevent the leaching of lipase[13,14] and also studied the influence of pore sizes of SBA-15 on lipase immobilization.[15] Considering that lipases are apt to adsorb on more or less hydrophobic supports and in some cases show high activity due to the interface activation,[16,17] we modify SBA-15 with hydrophobic alkyl groups via post-synthesis grafting and one-pot condensation, and prepare mesoporous silica-lipase hybrids in which porcine pancreas lipase (PPL) is chosen to be immobilized. The stabilizing effects of support surface on PPL are discussed in relation to the surface hydrophilicity/hydrophobicity. On the basis of the support properties and PPL structure, the adsorption mechanism is proposed and the influence of supports on the active conformation of lipase is speculated.

Experimental Section

Materials

Porcine pancreatic lipase (PPL, Sigma) was stored at 0–4°C and used without further purification. P123 ($EO_{20}PO_{70}EO_{20}$, molecular weight = 5800) from Aldrich, organic silanes from Hubei Huabang Chemical Company, and other reagents of analytical purity were all used as received.

Preparation of siliceous and alkylated-SBA-15

Siliceous SBA-15 was synthesized following the reported procedure.[18] P123 [0.345 mmol (2 g)] was dissolved in the mixture of 60 ml of deionized water and 9.55 ml of 12 M HCl aqueous solution. Thereafter, 21 mmol (4.43 g) of TEOS was added. The mixture was stirred at 45°C for 24 h, then transferred to a Teflon bottle, and heated at 100°C for 48 h. The white solid was filtered, washed with deionized water, and dried at ambient temperature. The template was removed by refluxing in ethanol (2 g of solid in 3 × 100 ml EtOH) for 8 h.

The siliceous SBA-15 solid prepared previously was washed well with acetone, then dried to remove all traces of water, and added to a solution of dimethyl (DM) dimethoxy silane (3.0 g for 0.5 g of SBA-15) in 30 ml of toluene under nitrogen atmosphere. Stirred at 80°C for 24 h, the resulting mixture was filtered. The solid was washed with cold acetone to remove residual toluene and silane, and dried to give the resulting support denoted SBA-DM. The grafting with diisopropyl (DIP) dimethoxy silane or diisobutyl (DIB) dimethoxy silane was performed following the same procedure, and the resulting solids were denoted SBA-DIP and SBA-DIB.

One-pot condensation synthesis of alkylated SBA-15 was performed using the same procedure as for siliceous SBA-15. Dimethyl (DM; 1 mmol) dimethoxy silane or diisopropyl (DIP) dimethoxy silane was introduced together with 20 mmol (4.26 g) of TEOS into P123 aqueous solution. The samples were named as is-SBA-DM and is-SBA-DIP, respectively. P123 was removed by refluxing in ethanol.

Immobilization of PPL

Support solid (0.1 g; SBA-15, SBA-DM, SBA-DIP, SBA-DIB, is-SBA-DM, or is-SBA-DIP) was added to the aqueous solution containing 0.025 g of PPL, 25 ml of pH 7.5 Tris-HCl buffer, and 25 ml of deionized water. The mixture was stirred at 30°C for 12 h. Thereafter, the solid was separated from the suspension by centrifugation and washed twice with Tris-HCl buffer. The amount of PPL immobilized on support was calculated by subtracting the lipase content in the supernatant from the total. The protein content was measured by Bradford method. Dried in air, the resulting bio-hybrids were denoted SBA-15-PPL, SBA-DM-PPL, SBA-DIP-PPL, SBA-DIB-PPL, is-SBA-DM-PPL, and is-SBA-DIP-PPL, respectively. In the measurements of adsorption isotherms, 50 mg of SBA-15, is-SBA-DM, or is-SBA-DIP was immerged in 5 ml of 0.5–7.0 mg/ml PPL solution, oscillated at 25°C for 12 h to achieve adsorption equilibrium.

Characterization

Powder XRD patterns were obtained on a Rigaku D/MAX-2500 X-ray diffractometer (Cu K_α radiation) with a scan rate of 0.5°/min. Transmission electron micrographs (TEM) were taken on a FEI Tecnai 20 electron microscope operating at 200 kV. The low-temperature N_2 sorption experiments were carried out on a Quantachrome Autosorb-1 system. The samples without PPL immobilized were outgassed at 200°C for 2 h, while the SBA-PPL hybrids were outgassed at 100°C for 4 h before measurements. The pore diameter distribution was calculated using the BJH method based on the desorption branch, and the surface area was calculated using the BET method based on the adsorption branch. The NMR spectroscopic investigations were performed on a Bruker AV300 NMR spectrometer at resonance frequencies of 75.5 MHz for ^{13}C MAS-NMR and 59.6 MHz for ^{29}Si MAS-NMR. Chemical shifts were referenced to TMS. The hydrophilic/hydrophobic property of support surface was characterized by water-contact angle measurements using JC2000A contact angle analyzer. SBA-15 support is pulverous and pressed disc method is not able to make an actual slick surface. So, the condition parameters such as the sample weight, disked pressure, and disked time are assured uniform in all measurements. At least three measurements are performed and the angles are reported as an average of all measurements. FT-IR spectra were obtained on a Bruker Vector 22 spectrometer using the standard KBr disk method. The spectra were smoothed by 13-point averaging before the Gauss curve-fitting (by Origin 8.0) was performed in the 1480–1700 cm^{-1} region. The centers of amide I and amine II were respectively fixed around 1640 and 1550 cm^{-1}. Thermal analysis was carried out using a BOIF instrument with a temperature programmed rate of 10°C/min.

Activity assays

The activity of PPL was determined through the hydrolysis of triacetin.[19] A mixture of 2.0 g of triacetin, 25 ml of

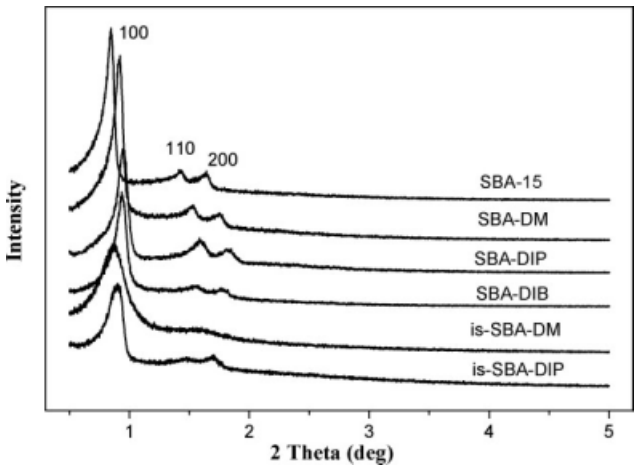

Figure 1. XRD patterns of siliceous and alkylated SBA-15.

Tris-HCl buffer, and 50 ml of deionized water was stirred for 30 min at room temperature to prepare a triacetin emulsion. Then 0.05 g of PPL or SBA-PPL hybrids was added. The mixture was continuously titrated with 0.1 M NaOH to maintain the pH constant at 7.5. The hydrolysis temperature was strictly controlled at 30°C. The volume of NaOH consumed in 30 min was recorded and the activity of PPL or immobilized PPL was calculated.

Stability tests

To compare the stability of soluble and immobilized PPL, all the samples were separately incubated under a certain condition. Then, the residual activity was determined by the titration procedure described above. The thermal stability was evaluated in two ways: PPL (immobilized or soluble) was incubated in Tris-HCl buffer (pH 7.5) for 1 h at 20°C, 30°C, 40°C, 50°C, and 60°C, respectively; or PPL (immobilized or soluble) was incubated in a Tris-HCl buffer (pH 7.5) for 5 h at 50°C. In the pH stability tests, PPL (immobilized or soluble) was incubated for 10 h in phosphate-citric acid buffer or carbonate buffer with pH of 5.0, 6.0, 7.0, 8.0, or 9.0. The sample was separated from the buffer and was washed twice with Tris-HCl buffer before activity assay. In the storage stability test, PPL (immobilized or soluble) was placed at room temperature (18°C) for a certain period and the activity was assayed every 5 days. The reusability test was done with four recycle runs. The SBA-PPL hybrids were recovered from the reaction system by centrifugation.

Results and Discussion

Siliceous and alkylated SBA-15

Figure 1 shows the XRD patterns of siliceous and alkylated SBA-15 solids. The siliceous SBA-15 material exhibits XRD pattern with three well-resolved peaks that can be indexed as (100), (110), and (200) reflections, consistent with the XRD pattern of a well-ordered hexagonal structure (*P6mm*).[18] The alkylated SBA-15 materials via post-synthesis grafting show similar XRD patterns to their grafted precursor, except that the intense (100) reflection shifts slightly to higher 2θ, indicating the mesoporous structures are well retained throughout the grafting process. For the one-pot modified SBA-15 (is-SBA-DM and is-SBA-DIP), the (100) reflection is relatively broad, and the (110) and (200) reflections are not very apparent. It seems that the alkylated SBA-15 via one-pot condensation has less-ordered long range structure. But in the TEM image shown in Figure 2, well-ordered 1D mesopores arraying along the long axis with the pore size of ~6 nm, rather similar to siliceous SBA-15, could be clearly revolved for one-pot alkylated SBA-15 materials. We have attempted to prepare diisobutylated SBA-15 by one-pot condensation but failed. The resulting material always shows amorphous phase unless the molar ratio of DIBDMS/TEOS is reduced to 1%.

The mesoporous features are evaluated by nitrogen sorption experiments and the pore structural parameters are shown in Table 1. The results indicate that the grafting modification causes no marked decrease in the pore diameter, surface area, and pore volume. The alkylated SBA-15 supports by one-pot condensation have higher surface area and pore volume whilst a little smaller pore sizes than by post-synthesis grafting. All the supports have similar pore

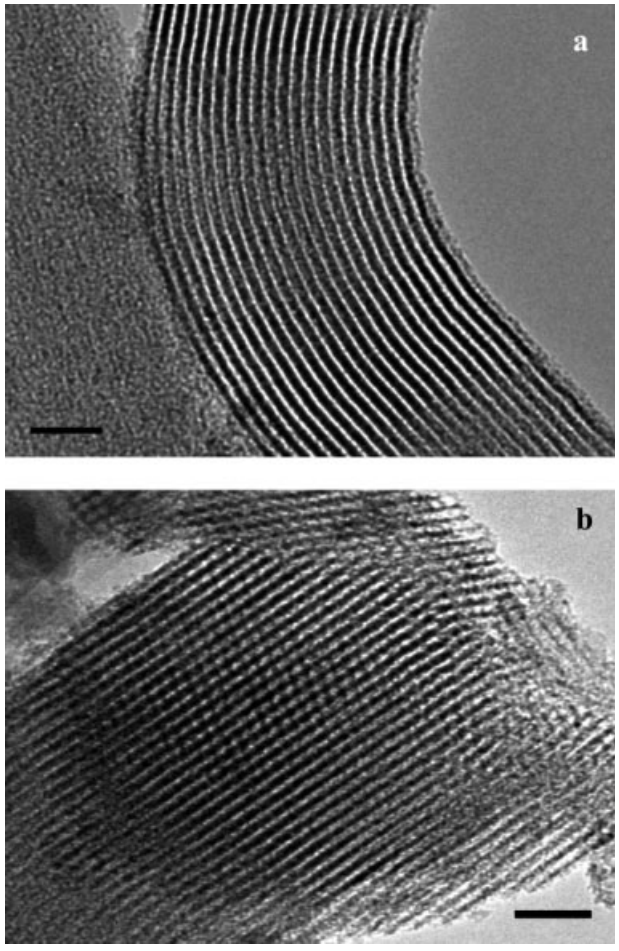

Figure 2. TEM images of (a) siliceous SBA-15 and (b) is-SBA-DIP.

Scale bars: 50 nm.

Table 1. Pore Structure Parameters and Organic Contents of SBA-15 Supports

Sample	Pore Diameter (nm)	Pore Volume (cm^3/g)	Surface Area (m^2/g)	Weight Loss (%)	Organic Content (mmol/g)
SBA-15	6.6	0.92	546	–	–
SBA-DM	6.6	0.83	465	8.81	2.4 [—CH_3]
SBA-DIP	6.6	0.83	504	8.94	0.9 [—CH—$(CH_3)_2$]
SBA-DIB	6.6	0.79	479	13.29	1.4 [—C_2H_5—$(CH_3)_2$]
is-SBA-DM	6.5	1.42	797	16.66	7.7 [—CH_3]
is-SBA-DIP	5.6	1.16	951	18.80	3.2 [—CH—$(CH_3)_2$]

diameters around 6 nm with narrow size distribution (from BJH desorption isotherm). This size is a nice match to PPL which has an average molecular diameter about 4–5 nm.[20]

In the ^{29}Si MAS NMR spectra illustrated in Figure 3, there are three resonance peaks at −92 ppm, −101 ppm, and −110 ppm attributed to Q^2, Q^3, and Q^4, respectively.[21] The obviously higher intensity of Q^3 than Q^4 shows a large amount of silanol groups existing on the pore surface. Compared with siliceous SBA-15, the post-synthesis alkylated samples display a decreased proportion of both Q^2 and Q^3 resonances relative to Q^4, due to the consumption of surface silanol hydroxyls in the grafting modification. The content of surface hydroxyl groups on one-pot alkylated SBA-15 is also lower than on siliceous SBA-15, because of the presence of surface alkyl chains introduced in the synthesis. In addition to the three resonances (Q^2, Q^3, and Q^4), one more peak appears for post-synthesis alkylated SBA-15 in the region of −15 to −25 ppm, which could be assigned to the silane silicons with T^2 linkage. Similarly, −14 ppm and −18 ppm are observed, respectively, in the NMR spectra of is-SBA-DM and is-SBA-DIP. The presence of T^2 resonance and the change in the relative intensity of framework —Si—O—Si— peaks confirm the alkyl-functionalization of SBA-15.

The ^{13}C CP/MAS NMR spectra of alkylated SBA-15 are shown in Figure 4. For SBA-DIP and is-SBA-DIP, a peak at 12.6 ppm is ascribed to the methylene carbons (Si—CH) and 15.7 ppm to methyl carbons (—CH_3) in isopropyl groups. For SBA-DIB, three peaks at 15.4 ppm, 23.5 ppm, and 25.3 ppm are observed, which are due to three carbons atoms in different position of Si—CH_2—CH—$(CH_3)_2$. A peak at −0.8 ppm in the is-SBA-DM spectrum could be attributed to the methyl carbons (Si—CH_3) bonded to the silicon atoms. Some remaining methoxy groups (59 ppm) are observed in all alkylated samples and the resonances at 66–77 ppm are due to trace amounts of P123 surviving from the ethanol extraction.[22,23]

The contents of organic moieties in SBA-15 supports were qualitatively determined by thermal analysis. The organic moieties display sharp weight loss beginning at 230°C and completely finishing at 650°C. As shown in Table 1, the one-pot condensation turns out higher organic content than the post-synthesis grafting. But it has to be noted that the alkyl groups introduced by one-pot condensation might be partially buried in the pore walls, rather than all located on the surfaces.

Lipase uptake and activity

The percentage of enzyme uptake (immobilization efficiency) and the activity of the resulting immobilized enzyme when the initial concentration of PPL is 0.5 mg/ml and the initial ratio of SBA-15 to PPL is 4:1 (wt.) are given in Table 2. The alkyl-functionalization increases the immobilization efficiencies and PPL contents on the support. The enhancement of PPL uptake by the alkylation becomes distinguished with the increase in the carbon chain length of

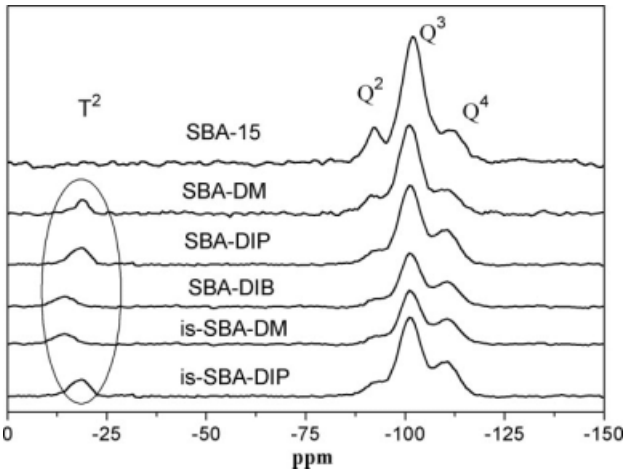

Figure 3. ^{29}Si MAS NMR spectra of siliceous and alkylated SBA-15.

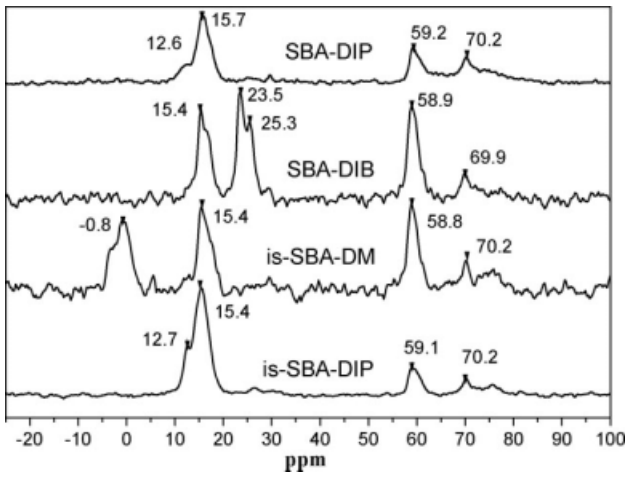

Figure 4. ^{13}C CP/MAS NMR spectra of siliceous and alkylated SBA-15.

Table 2. PPL Contents and Activities of SBA-PPL Hybrids

Support	Immobilization Efficiency*	PPL Content (mg PPL/g Support)	Activity (IU/g Bio-hybrids)	Relative Activity†
SBA-15	70.9%	177	12.0	36%
SBA-DM	71.2%	178	14.2	43%
SBA-DIP	77.3%	193	25.6	72%
SBA-DIB	83.3%	208	22.9	61%
is-SBA-DM	72.6%	182	24.0	71%
is-SBA-DIP	79.0%	198	28.5	79%

*The initial concentration of PPL solution is 0.5 mg/ml.
†The activity of soluble PPL is 219 IU/g.

organic moieties. The immobilization efficiency on dimethyl-functionalized support only shows a slight increase compared to siliceous SBA-15 (Table 2), despite the methyl moiety is incorporated with silica in higher amount than isopropyl and isobutyl groups (Table 1). To further demonstrate this observation, the initial concentration of PPL was increased from 0.5 mg/ml to 5 mg/ml and the initial ratio of SBA-15 and PPL was changed to 2:1, then the immobilization was performed (Table 3). The dependence of immobilization efficiency on the support surfaces becomes more evident as can be seen in Table 3. The is-SBA-DIP has a noticeable higher uptake percentage than is-SBA-DM and SBA-15. The PPL content is 229, 270, and 366 mg, respectively, on per gram of SBA-15, is-SBA-DM, and is-SBA-DIP. The adsorption isotherms of PPL, shown in Figure 5, also illustrate the difference between SBA-15, is-SBA-DM, and is-SBA-DIP as supports in the adsorption capacity. The result agrees well with the immobilization efficiency and PPL content measured previously. The organic functionalization of support surface favors PPL adsorption. The longer is the carbon chain of functional group, the higher is the adsorption capacity for PPL.

The difference in adsorption amount and capacity for PPL could be rationally explained with the difference in the surface hydrophilicity/hydrophobicity. As shown in Table 3, the contact angles indicate that all the support surfaces are hydrophilic due to the existence of silanol groups that have been proved by ^{29}Si MAS NMR. But as expected, the alkylated SBA-15, especially is-SBA-DIP, has a less hydrophilic surface than siliceous SBA-15.

Additionally, there is no noticeable difference observed (Table 2) in the immobilization efficiencies when the supports are alkylated with the same organic moiety, although the organic moiety is introduced in higher content by one-pot condensation than by post-synthesis. This is supposed to be associated with the location of organic moieties. As mentioned previously, the organic groups might be partially buried in the one-pot condensation.

Figure 6 shows the N_2 adsorption–desorption isotherms of SBA-15 supports before and after PPL immobilization. The PPL immobilization hardly changes the features of type IV isotherm and H1 hysteresis loop. But the detailed changes of pore structural parameters, shown in Table 3, indicate that the PPL immobilization leads to reduction in both surface area and pore volume. This reduction is more noticeable for alkylated supports, consistent with their higher PPL contents. The PPL immobilization also causes the pore diameter at maximum distribution to shift to small size in 1.6–1.8 nm. The PPL has not been adsorbed in full monolayer coverage. So, the PPL immobilization could not cause the pore diameter to reduce in the size of one PPL molecule.

The different percentage of enzyme uptake on siliceous and alkylated SBA-15 could be explained by the adsorption mechanism of PPL. The non-covalent forces existing between the PPL and supports include hydrogen bonding, electrostatic and hydrophobic interactions. The large amount of Si—OH or Si—O^- groups on the pore surface can form hydrogen bond with the amino and carboxylic groups in PPL. In fact, hydrogen bonding is not the dominating factor while electrostatic interactions and hydrophobic interactions are regarded more important for protein adsorption.[4,24–26] A lower uptake amount of PPL on siliceous SBA-15 is due to the charge repulsion between PPL and support surface. Both SBA-15 (pI $\cong$ 3.6) and PPL (pI = 5.0) are electronegative when the immobilization is carried out at pH = 7.5. This repulsion can be weakened by the surface alkyl-functionalization. In addition, PPL, although water-soluble, has hydrophobic pockets on its surface and the surrounding of its catalytic site.[17] Dimethyl, diisopropyl, and diisobutyl moieties attached to the surface can interact with PPL via hydrophobic interaction, which favors the enzyme uptake.

Many cases of lipase adsorption on various hydrophobic supports have been reported.[3,16,17,27] But the results could be quite different. Sometimes lipase is hyper-activated or in some cases it is completely inactive. So, it is necessary to discuss the hydrophobic interactions further. Some possible

Table 3. The Change (Δ) of Pore Structure Parameters in High PPL Loading

Support	θ (°)	Immobilization Efficiency*	PPL Content (mg PPL/g Support)	Δ (Pore Diameter) (nm)	Δ (Pore Volume) (cm^3/g)	Δ (Surface Area) (m^2/g)
SBA-15	25	45.8%	229	1.6	0.31	114
is-SBA-DM	31	54.0%	270	1.6	0.80	371
is-SBA-DIP	63	73.1%	366	1.8	0.75	541

*The initial concentration of PPL solution is 5 mg/ml.

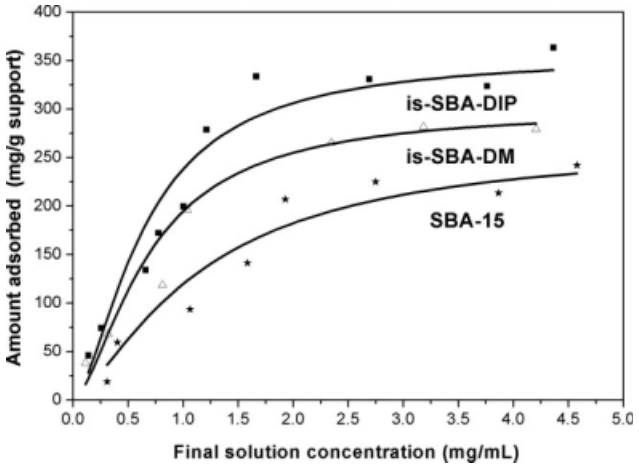

Figure 5. PPL adsorption isotherms on SBA-15, is-SBA-DM, and is-SBA-DIP at pH 7.5.

The solid lines are simulated curves using Langmuir model based on the experimental data.

mechanisms have been proposed and the point is whether involving the active site or not.[17] PPL molecule contains the active site with a catalytic triad formed by Ser153, Phe216, and His264.[28] The active site is covered by a surface loop consisting of a short one-turn α-helix with a tryptophan residue. The lid is shifted, exposing the active site when substrate accesses and reaction occurs.[29] In addition to the hydrophobic groups distributing on the lipase surface, the active site and the back side of the lid as well as the loop are all hydrophobic. If the adsorption involves the catalytic triad, it may lead to completely inactive, while if the adsorption involves the loop or lid, it may result in interfacial activation, which would give a remarkably high relative activity.[16] Neither of the two situations occurring, it may just be thought as a conventional hydrophobic adsorption. The interfacial adsorption between the alkylated-SBA-15 and PPL should be assigned to the last situation because neither marked inactivation nor activation is observed according to the relative activity shown in Table 2.

The conventional hydrophobic adsorption is not supposed to change the secondary structure of PPL. So, the FT-IR spectra of amide I and II regions are used to study the protein conformation to make it confirmed. The amide I band near 1650 cm^{-1} is due to the C=O stretching mode. The amide II band near 1550 cm^{-1} is due to the bending and the stretching mode of N—H and C—N vibrations. Typically, the disappearance of amide II band is used to confirm the protein unfolding.[30,31] Figure 7 (curve a) displays the FT-IR spectrum of soluble PPL with amide I band centered at 1640 cm^{-1} and amide II band at 1550 cm^{-1}. The amide III band is not discussed here for it is overlapped by the strong broad band of framework Si—O—Si asymmetric stretching vibrations. The intensity ratio of amide I to amide II band for the PPL adsorbed on is-SBA-DIP support (curve b) is similar to the pristine PPL, indicative of no change of the protein secondary structure in the immobilization.

As can be seen from Table 2, the activity and relative activity of PPL immobilized on alkylated supports are all higher than on siliceous SBA-15, indicating that the hydrophobic interaction favors the PPL activity, consistent with observed previously.[15] But the activity promotion is not so high as observed previously[15] in a less PPL content (~100 mg/g support), which could well explained by the enhanced diffusion restriction brought about by high PPL loadings. The hydrolytic reaction catalyzed with PPL is carried out in the emulsion of triacetin, while soluble PPL can exist at the

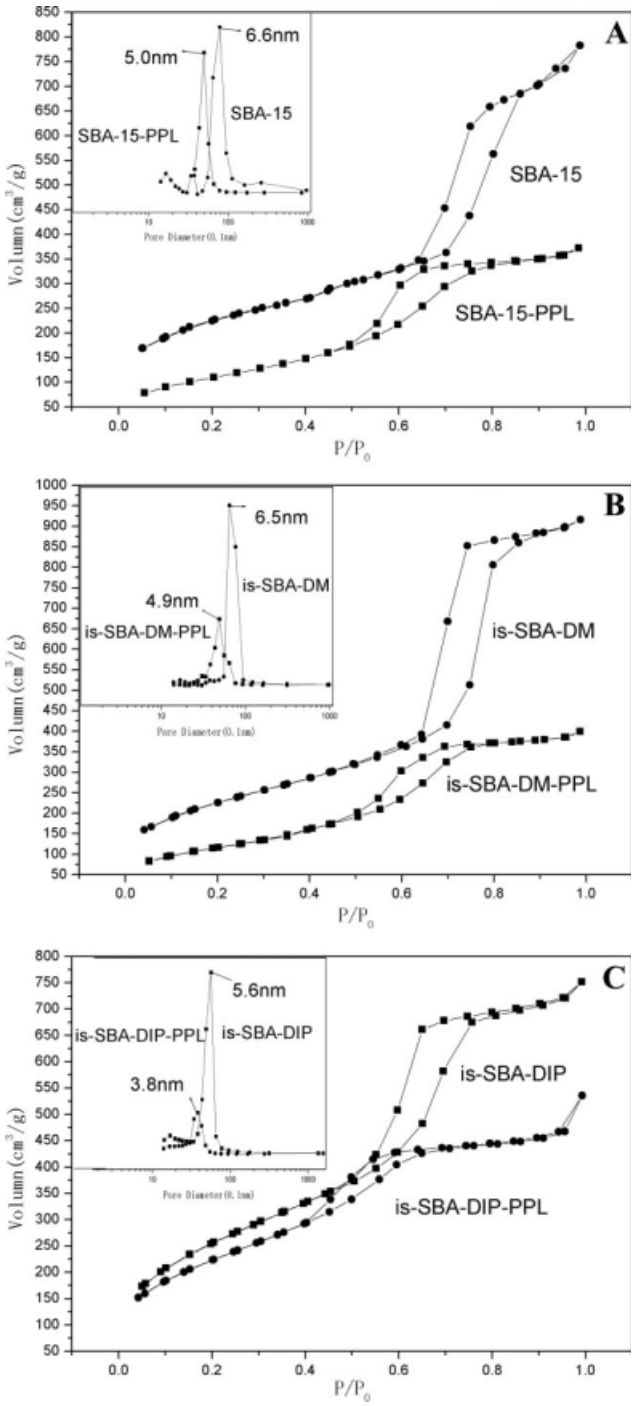

Figure 6. N_2 sorption isotherms and pore size distribution (inset) of (A) SBA-15, (B) is-SBA-DM, and (C) is-SBA-DIP before and after PPL immobilization.

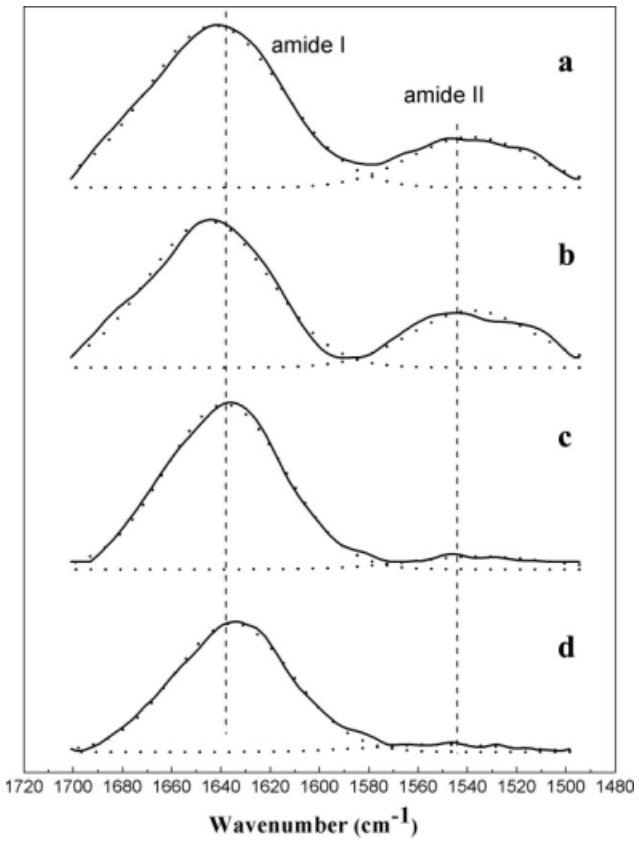

Figure 7. The amide I, II region in the FT-IR spectra of (a) PPL, (b) is-SBA-DIP-PPL, (c) incubated is-SBA-DIP-PPL at pH = 5, and (d) incubated is-SBA-DIP-PPL at pH = 10.

oil–water interface and directly contact with substrate. PPL immobilized in the pores requires the substrate to diffuse to access active sites. Higher PPL loading results in higher channel tortuosity, so that the diffusion resistance of the substrate largely increases and even some PPL become inaccessible, which causes the decrease in the apparent activity.

Activity stabilities

Both the intrinsic stability and operational stability are important indexes for biocatalysts. The thermal stability, pH stability, and storage stability of SBA-PPL hybrids are investigated in this work by monitoring the residual activity (normalized by each initial enzyme activity). The reusability was also studied.

Thermal Stability. As shown in Figure 8, soluble PPL maintains around 85% of the initial activity after 1 h incubation at 30°C and the activity halves at 50°C. When the temperature is elevated to 60°C, PPL loses nearly 90% of its initial activity. All of the immobilized PPL shows higher thermal stability than soluble PPL. SBA-15-PPL exhibits a gradual activity decrease with incubation temperature, similar to soluble PPL. But more than 40% of its initial activity is retained even after thermal treatment at 60°C. The activity of is-SBA-DM-PPL begins to decline above 40°C, while no visible loss in the activity is observed till 60°C for is-SBA-

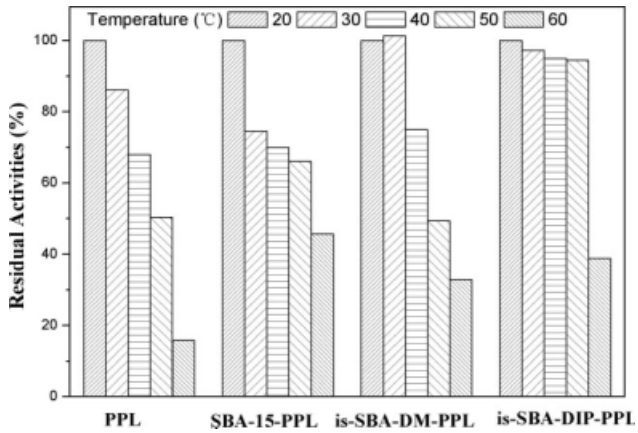

Figure 8. Residual activities of soluble PPL and SBA-PPL hybrids incubated for 1 h at different temperatures.

DIP-PPL. The residual activities of soluble PPL and immobilized PPL in 5 h incubation at 50°C have additionally been assayed. Soluble PPL retains 6% of its initial activity, SBA-15-PPL of 11%, is-SBA-DM of 13%, and is-SBA-DIP-PPL of 37%. This result also indicates that SBA-PPL hybrids have higher thermal stability. What's more, more hydrophobic support, in our experiment, shows better improvement of PPL resistance to temperature exposure.

pH Stability. Figure 9 illustrates the effect of pH on the PPL activity. The incubation medium with a pH of 7.0 favors the activity of both soluble and immobilized PPL. A higher or lower pH value causes the activity to decline. The deviation of pH from 7.0 results in more obvious decrease in the activity of immobilized PPL than soluble PPL, indicating that the immobilization boosts the pH sensitivity of PPL. Moreover, the PPL adsorbed on the support surfaces with alkyl chains attached is more sensitive to pH than on siliceous SBA-15 with only surface silanol groups.

Storage Stability. Lipase is generally stored at 0–4°C for refrigerant temperatures make for the stability of nature conformation. However, in industrial application, enzyme is sometimes ineluctably exposed to the external environment.

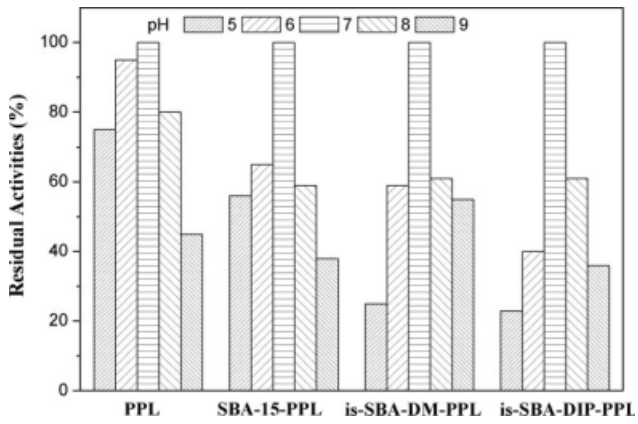

Figure 9. Residual activities of soluble PPL and SBA-PPL hybrids incubated for 10 h under different pH.

Enzyme is apt to inactivation rapidly when stored at room temperature. So, the storage stability has always been of concern for immobilized enzyme. Figure 10 illustrates the residual activities of PPL stored at ambient temperature (18°C) for a certain period. The activity of PPL immobilized on siliceous SBA-15 shows similar decrease to that of soluble PPL with extension of storage period. But the storage stability has been enhanced to different extents by the immobilization, especially on the supports modified with DIP moiety. For example, in 10 days, the residual activity of soluble PPL is 32%, while that of SBA-15-PPL, is-SBA-DM-PPL, and is-SBA-DIP-PPL is 48%, 59%, and 72%. The half life of soluble PPL at room temperature is about 8 days while that of SBA-15-PPL, is-SBA-DM-PPL, and is-SBA-DIP-PPL is extended to 10, 13, and 16 days.

Reusability. Figure 11 presents the activities of SBA-15-PPL, SBA-DM-PPL, SBA-DIP-PPL, and SBA-DIB-PPL in recycles. The activity of PPL immobilized on siliceous SBA-15 (SBA-15-PPL) exhibits an obvious decrease in the first reuse and then maintains around 40% of its initial activity, while that on functionalized supports show a gradual decrease along with recycle number. Especially for SBA-DIB-PPL, it still remains more than 50% of its initial activity after being recycled for 4 runs.

The stability tests indicate that the immobilized PPL has higher thermal ability and storage stability than soluble PPL, and the alkyl-functionalized supports show better performance than bare SBA-15. This is not surprising because it has been found[32,33] that confining enzyme in the channels of mesoporous supports improves its intrinsic stability. Besides the confinement of the pores matching the PPL size, the hydrophobic surfaces of the alkylated supports impel the residual water to PPL, protecting PPL from denaturation as well. However, the immobilization makes PPL more sensitive to pH change. Generally, extreme pH even leads to irreversible inactivation as a result of protein unfolding. As can be observed from Figure 7 (curves c and d), the amine II band of is-SBA-DIP-PPL almost disappears after incubation at pH 5.0 and 10.0. The corresponding amide I/amide II intensity ratio increases from 3, the value of native PPL, to 11 and 14. The disappearance of amide II N—H stretching mode indicates the occurrence of protein unfolding.[30] So, it

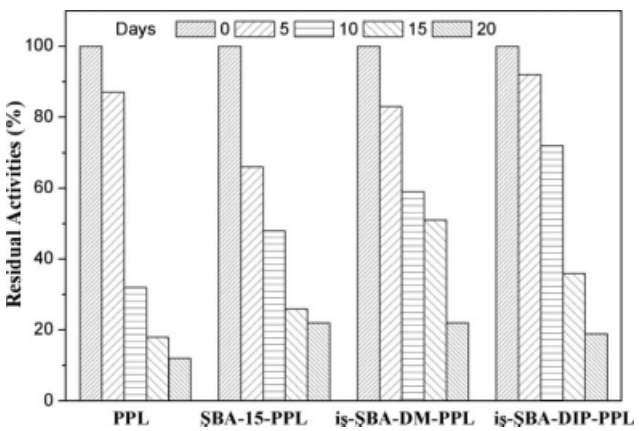

Figure 10. Residual activities of soluble PPL and SBA-PPL hybrids stored at 18°C for days.

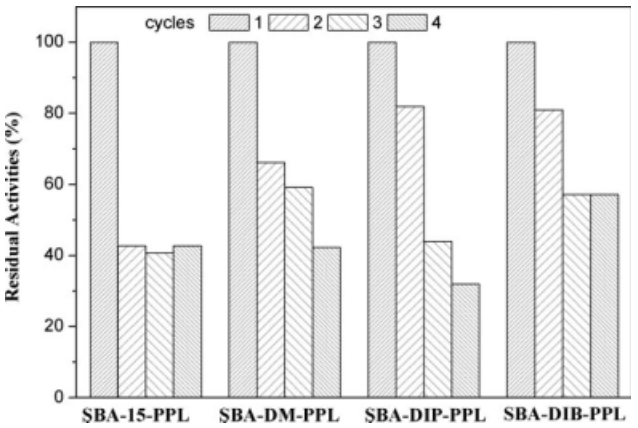

Figure 11. Residual activities of SBA-15-PPL, SBA-DM-PPL, SBA-DIP-PPL, and SBA-DIB-PPL in four recycle runs.

is supposed that PPL dissociates to intermediate states in different pH buffers, and the dissociation is partially irreversible when they were put back into the pH 7.5 buffer. The supports are likely to maintain the intermediate states, and therefore, cause more irreversible inactivation.

To confirm the reason for the activity loss caused by incubation at elevated temperature and varied pH, the supernatants of incubated SBA-PPL solids were tested to measure the protein contents. Only 5%, 3%, and less than 1% of PPL were leached from is-SBA-DIP-PPL in the incubation at 60°C (for thermal stability test), pH 10 and pH 5 (for pH stability test). The value is negligible compared to the percentage of activity loss.

Conclusion

In summary, compared to siliceous SBA-15, the alkylated SBA-15 supports show higher immobilization capacity for PPL via hydrophobic adsorption. The hydrophobic interaction doesn't involve the PPL active site. Therefore, neither hyper-activation nor total inactivation occurs. There is no evident difference observed in the immobilization efficiency between the supports modified by the same organic moiety in two methods. The investigation of the intrinsic stability of PPL indicates that the entrapment in mesoporous supports makes PPL more resistant to thermal exposure but more sensitive to pH values. The organic modification inhibits the enzyme leaching to some extent, resulting in higher residual activities in the operation recycles.

Acknowledgments

The authors thank the financial support from NSFC, Program for Changjiang Scholars and Innovative Research Team in University (IRT0406), Project 111 (B07004), and 973 Project (2009CB939802).

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Manuscript received Feb. 20, 2008, and revision received May 2, 2009.