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Bioreactors Based on Monolith-Supported Ionic Liquid Phase for Enzyme Catalysis in Supercritical Carbon Dioxide

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Abstract: Bioreactors with covalently supported ionic liquid phases (SILP) were prepared as polymeric monoliths based on styrene–divinylbenzene or 2-hydroxyethyl methacrylate–ethylene dimethacrylate, and with imidazolium units loadings ranging from 54.7 to 39.8 % wt IL per gram of polymer. The SILPs were able to absorb *Candida antarctica* lipase B (CALB), leading to highly efficient and robust heterogeneous biocatalysts. The bioreactors were prepared as macroporous monolithic mini-flow systems and tested for the continuous flow synthesis of citronellyl propionate in supercritical carbon dioxide ($scCO_2$) by transesterification. The catalytic activity of these mini-flow-bioreactors remained practically unchanged for seven operational cycles of 5 h each in different supercritical conditions. The best results were obtained when the most hydrophobic monolith, M-SILP-**8**-CALB, was assayed at 80 °C and 10 MPa, reaching a total turnover number (TON) of 35.8×10^4 mol product/mol enzyme. The results substantially exceeded those obtained for packed-bed reactors with supported silica-CALB-Si-4 catalyst under the same experimental conditions.

Keywords: continuous processes; enzyme catalysis; green chemistry; supercritical fluids; supported ionic liquids

Introduction

Supercritical carbon dioxide ($scCO_2$) is an excellent 'green' solvent with a huge potential for both the synthesis and separation of chemicals. It represents an interesting alternative to organic solvents for developing cleaner and more environmentally friendly chemical processes, because it provides a clean, non-toxic, non-flammable and tuneable solvent system, which is easily removed to leave reaction products free from undesirable organic residues.[1] The greenness of chemical transformations in $scCO_2$ should also concern the applied catalyst.[2] Thus, one of the most straightforward "green" practical applications of $scCO_2$ is its combined use with enzymes. Since the pioneering work of Randolph et al. and Hammond et al.[3] in 1985, $scCO_2$ has been described as a solvent with a strong deactivating effect on enzymes, which is the main drawback for application in industrial biocatalytic processes.[4,5] Several strategies have been developed to stabilise the enzyme (e.g., covalent attachment on supports coated with hydrophilic polymers, entrapment in silica-aerogels, cross-linking enzyme aggregates, etc.).[6,7] However, the best results for enzyme catalysis in $scCO_2$ have been obtained when ionic liquids (ILs) were applied as liquid immobilisation supports for the enzymes.[8] In this context, biocatalytic IL/$scCO_2$ biphasic systems, based on enzymes retained into the IL phase (catalytic phase), while substrate and product reside largely in the $scCO_2$ (extractive phase), were the first attractive and efficient approach for continuous green processes.[8a,b] The advantages to use of ILs as additives or coating material to enhance lipase-catalysed transesterification in either organic solvents or supercritical fluids have also been described.[9] In this way, ILs have emerged as exceptionally interesting non-aqueous reaction media for enzymatic transformations mainly

due to their ability to maintain a high level of activity, stereoselectivity, and stability of enzymes in chemical transformations,[10] even under extremely harsh conditions (e.g., $scCO_2$ at 150 °C and 100 bar).[11] The physical properties of ILs (density, viscosity, melting point, polarity, etc) can be finely tuned by appropriate structure selection. Their interest as green solvents resides in their high thermal stability and very low vapour pressure, which mitigates the problem of the emission of volatile organic solvents to the atmosphere.[12]

Although ILs have become commercially available, they are still relatively expensive compared with traditional solvents. Besides, some of them show evidence of low biodegradability and (eco)toxicological properties.[13] Hence, the immobilisation of ILs onto a support or structured material (e.g., by simple impregnation, covalent linking of the cation, sol-gel method, etc.) is a highly attractive strategy to minimise the amount of ILs used, while maintaining their catalytic properties. Additionally, supported ILs have the advantage of easy separation and recyclability as well as a potential for use in the development of continuous processes.[14]

Recently, we reported the preparation of supported ionic liquid phases (SILP) as monolithic rods by the surface modification of macroporous polystyrene–divinylbenzene (PS-DVB) resins.[15] As this type of material is not able to adsorb ILs as films onto the surface, unlike silica gel,[14] our approach was based on immobilisation by the covalent binding of IL-like units (alkylimidazolium cations) to the polymer surface. In this way, IL properties are transferred to the solid phase, leading to a monolith-supported ionic liquid phase (M-SILP) able to be used to develop mini-flow reactors for continuous processes, which offer many potential advantages from an industrial point of view over existing batch techniques or packed-bed reactors containing particulate catalysts.[16]

In this paper, we develop a new generation of highly efficient enzymatic reactors with suitable mechanical stability for continuous-flow processes in $scCO_2$ (Figure 1). The catalytic system is based on an enzyme (*Candida antarctica* lipase B, CALB) immobilised by non-covalent attachment onto macroporous M-SILPs, which were applied for a continuous transesterification process in $scCO_2$. The synthesis of citronellyl propionate (**3**) from vinyl propionate (**1**) and citronellol (**2**) catalysed by CALB was selected as reaction model (Scheme 1). This approach combines the advantages of supported ionic liquid as a "supported liquid solvent", those of an immobilised enzyme as a "green" catalyst and the use of a supercritical fluid as a "green" reaction/extraction solvent with the advantages of a continuous flow process, easy product separation and catalyst reuse.

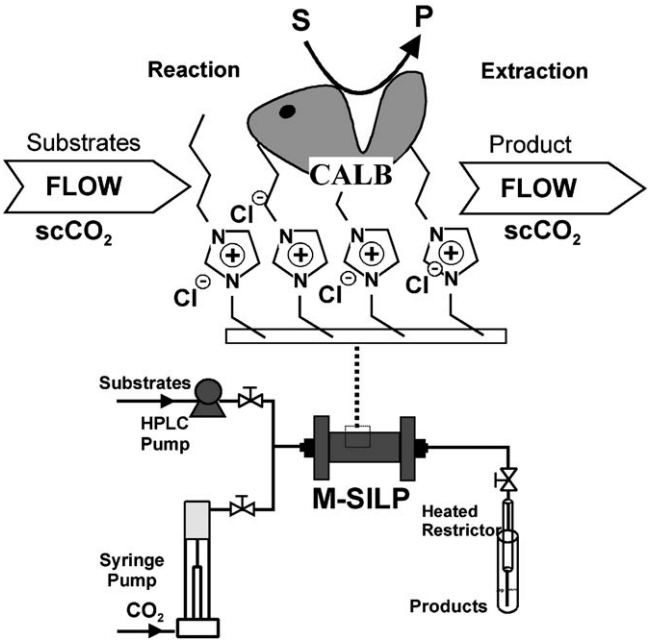
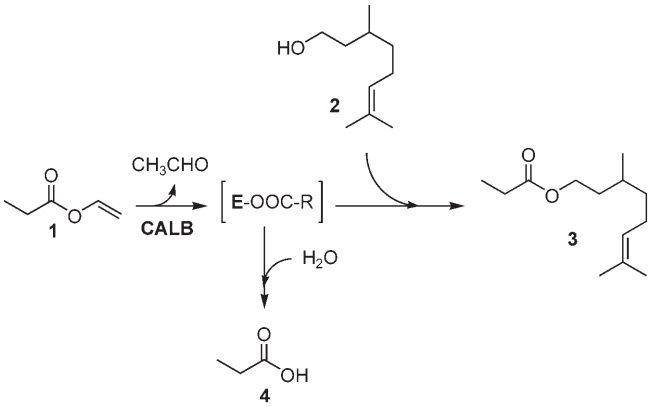


Figure 1. Set-up of the reactor with immobilized CALB onto monolith-supported ionic liquid phase for continuous operation under flow conditions in $scCO_2$.

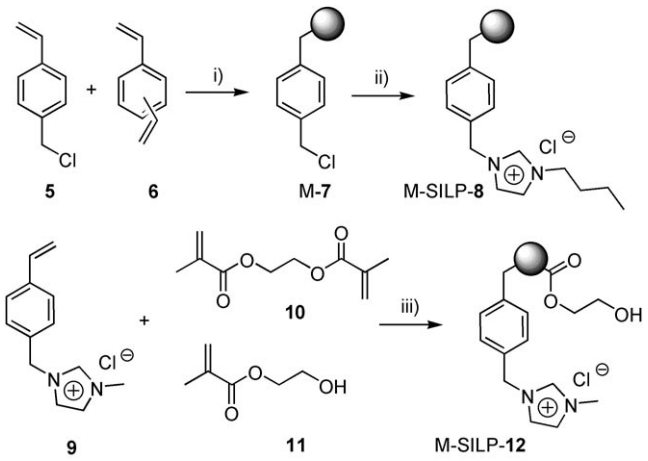


Scheme 1. Kinetic mechanism for CALB-catalysed citronellyl propionate (**3**) synthesis from vinyl propionate (**1**) and citronellol (**2**) by transesterification.

Results and Discussion

Synthesis and Characterisation of the Monolith-Supported Ionic-liquid Phase (M-SILP)

The preparation of the polymeric monolithic materials is depicted in Scheme 2. Monolithic polymer (M-**7**) was synthesised by thermally induced radical solution polymerisation of a monomeric mixture of *p*-chloromethylstyrene (**5**) and divinylbenzene (**6**), using toluene/1-dodecanol as the precipitating porogenic mixture and AIBN as the radical initiator (Table 1). In order to take full advantage of the potential of these monolithic systems, polymerisation was carried



Scheme 2. Synthesis of monolith-supported ionic liquid phase M-SILP-**8** and M-SILP-**12,** i) 30% (w/w) of a mixture **5:6** (40:60 by weight) in a porogen mixture (75:25 dodecanol:toluene by weight) 1% AIBN, 70°C, 24 h. ii) butylimidazole, 80°C. iii) 40% (w/w) of a mixture **9:10:11** (40:40:20 by mol) in DEG, 1% AIBN, 70°C, 24 h.

out within a stainless steel cartridge, which can be adapted to our supercritical reactor allowing us to use it as a mini-reactor (1 mL volume) under flow conditions (Figure 1). The monolithic-supported ionic liquid phase (M-SILP-**8**) was obtained by the post alkylation of M-**7** with *N*-butylimidazole. The introduction of the imidazolium IL-like moieties was monitored by the disappearance of the CH_2–Cl group, using both the FT-IR–Raman and a colorimetric method based on the NBP test.[17] A 95% conversion of the chloride groups was achieved after 5 h of reaction. Longer reaction times did not improve the conversion degree (Figure 2). Most likely, a small percentage of the CH_2–Cl groups are located in non-accessible highly cross-linked regions. The FT-Raman of M-SILP-**8** showed bands characteristic of 1-butyl-3-*p*-methylstyrene–imidazolium chloride (1505, 1345, 1078 and 1029 cm^{-1}, Figure 2). Elemental analysis showed the loading of IL to be 1.98 mequivs. of imidazolium units per gram of polymer, which it is equivalent to 54.7% of IL by weight of polymer (Table 1). In order to check if the microenvironment of the monolith was modified by attachment of the IL-like units, the shift experienced by a solvatochromic fluorescent probe, in this case pyrene, was studied.[18] The pyrene value I_I/I_{III} measured for M-SILP-**8** clearly indicated a completely different microenvironment sensed by excited-state pyrene. The pyrene I_I/I_{III} value was 1.44 for M-SILP-**8**, which is significantly higher than that measured for M-**7** ($I_I/I_{III}=1.01$) and similar to that observed in methanol ($I_I/I_{III}=1.33$).[18]

Since these macroporous polymers are designed to be used in the flow-through mode, they should be materials with a low flow resistance. Figure 3 shows that for polymer M-SILP-**8** the back pressure (normalised to a length of 1 cm) was lower than 0.2 MPa cm^{-1} for different flow rates (0.1–3.5 mL/min of CO_2), temperatures and pressures, confirming the good pressure resistance of this material to work as a packed-bed reactor under $scCO_2$. The low pressure drop observed with the increase in flow can be ascribed to the formation of monolithic polymers with a very high pore size, as revealed by the SEM image (showing pores larger than 1 μm, Figure 3). The resulting monolithic columns had similar structural properties to those that have been used for chromatographic applications and for other catalytic applications.[19,20]

Polymer M-SILP-**12** was synthesised by polymerisation of the corresponding monomeric ionic liquid [1-(*p*-vinylbenzyl)-3-methylimidazolium chloride, **9**][21] with ethylene dimethacrylate (EDMA, **10**) and 2-hydroxyethyl methacrylate (HEMA, **11**) and using DEG as poronic agent.

The enzyme [*Candida antarctica* lipase B (CALB)] was immobilised by simple adsorption of an aqueous solution of CALB. The resulting enzymatic catalysts (M-SILP-**8**-CALB and M-SLIP-**12**-CALB) showed a very high ionic liquid/enzyme ratio, which ensured full interaction between the protein molecule and the IL phase, and could favour enzyme stabilisation

Table 1. Characteristics of monolithic columns prepared according Scheme 2.

Entry	Monomeric composition (% wt.)[c]					ILs loading		Enzyme loading	IL/E
	5	**6**	**9**	**10**	**11**	mequiv. IL/g supp.[d]	g IL/g supp. (%)[e]	μmol E/g supp.[f]	ratio
1[a]	40	60	–	–	–	1.98	54.7	0.31	6387
2[b]	–	–	40	40	20	1.70	39.8	0.31	5484

[a] The porogenic mixture was toluene/1-dodecanol (1:3 w/w) with a 70:30 (w/w) porogens/monomers ratio.
[b] The porogen used was DEG mixed with monomers in a 60:40 (w/w) porogen/monomer ratio.
[c] The polymerisation was performed at 70°C by using 1% AIBN.
[d] Determined by elemental analysis.
[e] Calculated as percentage of IL moieties per gram of support, considering 1-butyl-3-*p*-methylstyrene-imidozolium chloride (Mw = 276.14 g mol^{-1}) as the IL moiety for M-SILP-**8**, and 1-methyl-3-*p*-methylstyrene–imidozolium chloride (Mw = 234.09 g mol^{-1}) the IL moiety for M-SILP-**12**, respectively.
[f] Mw for CALB was 33 kDa[4e].

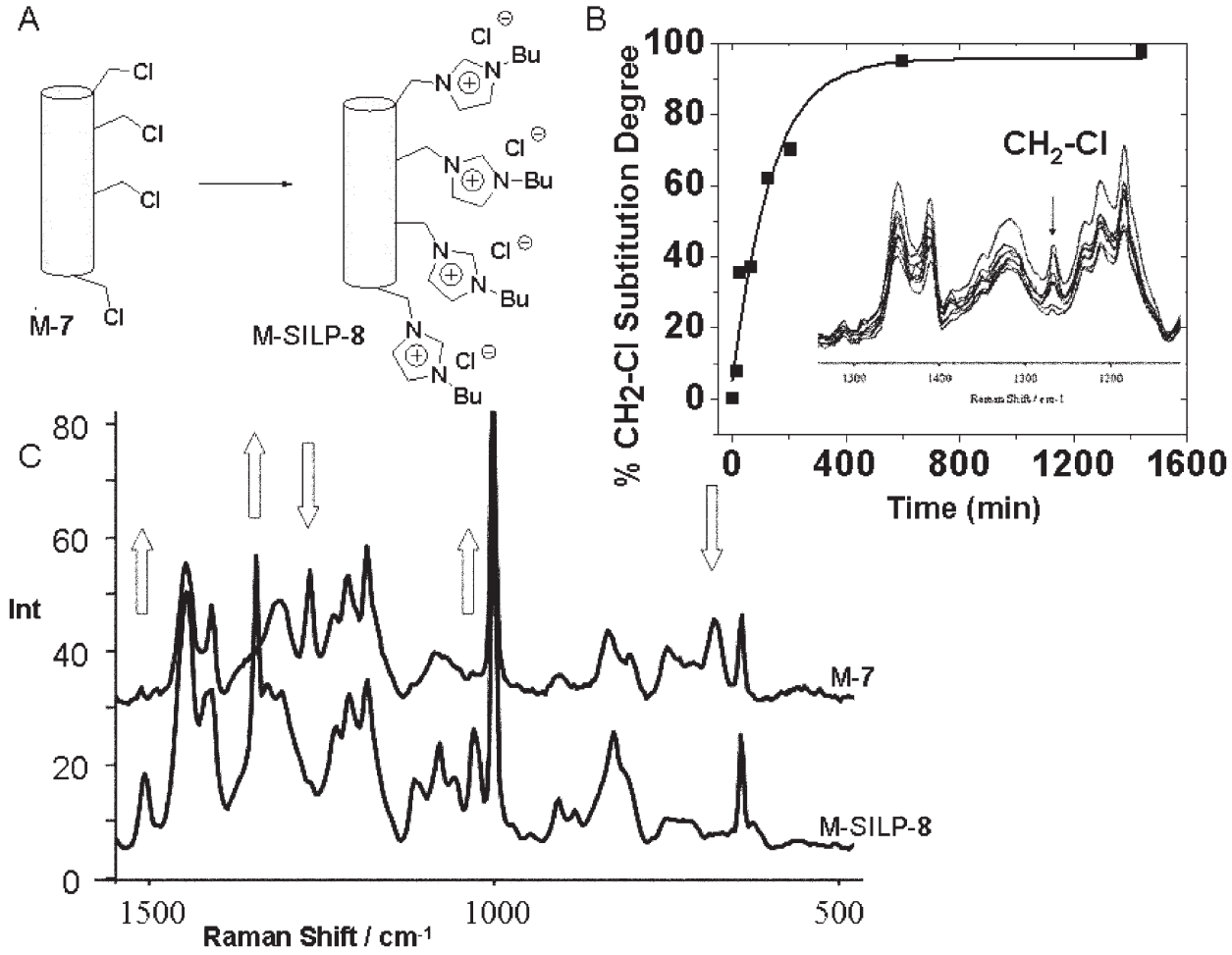


Figure 2. A: Synthesis of monolith-supported ionic liquid phase. **B:** Profile of substitution of CH_2−Cl groups by butylimidazole units with time, following the disappearance of the CH_2−Cl characteristic band at 1265 cm^{-1} of the FT-Raman spectra. (Inset shows an expansion in the 1300–1200 cm^{-1} region for the FT-Raman spectra of **M-7** at different reaction times). **C:** FT-Raman spectra expansion from 1600–500 cm^{-1} for M-**7** and M-SILP-**8.**

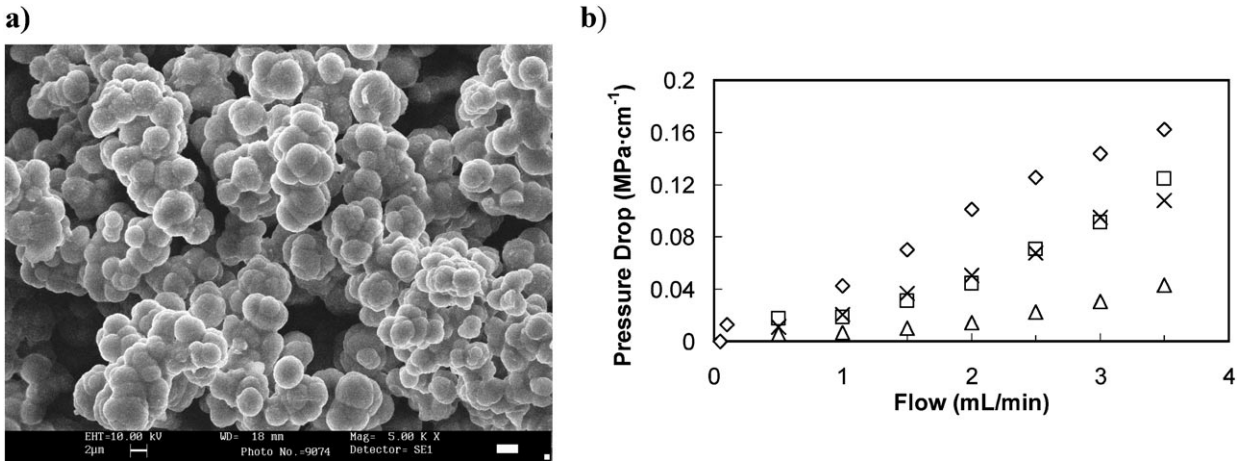


Figure 3. a) Micrograph of M-SILP-**8** obtained by scanning electron microscopy. **b)** Pressure drop (ΔP, MPa cm^{-1})·for M-SILP-**8** (normalised to a length of 1 cm) *vs.* CO_2 flow rate (mL min^{-1}) [(◇) 40 °C, 8 MPa, ρ = 0.311 g/mL. (□) 100 °C, 10 MPa, ρ = 0.191 g/mL. (×) 100 °C, 15 MPa, ρ = 0.336 g/mL. (△) 100 °C, 8 MPa, ρ = 0.143 g/mL.]

(Table 1).[22] M-SILPs not only provide high flow characteristics and mechanical stability, based on the intrinsic properties of these macroporous materials, but also an easy and simple protocol to anchor the enzyme onto a modified matrix with "enzyme friendly" moieties like alkyl-imidazolium cations.[23]

Synthesis of 3 by CALB-Monolithic Catalytic Bioreactors in $scCO_2$ under Continuous Flow Conditions

The ability of each monolith-SILP-CALB derivative to catalyse transesterification reactions in $scCO_2$ was tested by using the synthesis of **3** as the activity test (see Scheme 1, Figure 1). Terpene esters, such as **3**, are very important flavour and fragrance substances, and they may be considered natural when produced enzymatically.[24] For comparison, another CALB immobilised derivative, obtained by the adsorption of the enzyme onto a modified silica gel with butyl side chains (Si-C_4) was also studied.

Figure 4 depicts all the productivity profiles for the synthetic product **3** catalysed by each immobilised derivative obtained in continuous operation under different supercritical conditions (see Table 2). For each assayed condition, the catalyst operated in daily cycles (5 h/d of synthetic process and 19 h of storage under dry conditions) for two days, in order to observe both the level of activity, as well as any deactivation process that might take place.

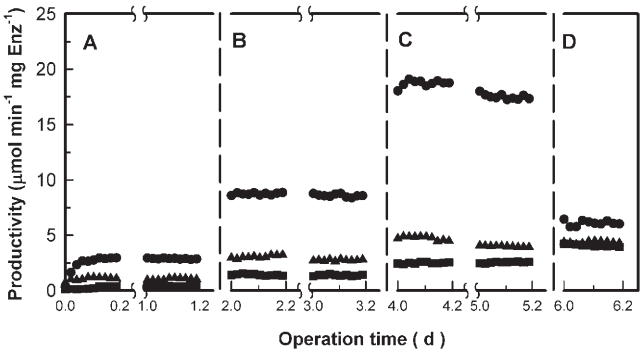


Figure 4. Productivity profiles for synthesis of **3** catalyzed by M-SILP-**8**-CALB (●), M-SILP-**12**-CALB (▲) and silica-butyl-CALB (■) with operation time, in a continuous process in the different assayed conditions (see Table 2 for experimental conditions **A**, **B**, **C** and **D**).

It can be seen that, for all the immobilised derivatives, productivity in the synthetic process increased with temperature, reaching a maximum level at 80 °C, decreasing at 100 °C. Temperature seems to be an activating parameter for enzymatic catalysis, although, in the case of $scCO_2$, a decrease in the fluid density has also been described as a positive parameter in enzyme activity (see Table 2).[6,7,24] In this regard, the activation of a commercial immobilised CALB preparation (Novozym 435) by temperature in different supercritical fluids (ethane, propane and CO_2) for ester synthesis has also been described.[22a] This effect was directly related with the decrease in fluid density, which produced a reduction in the internal diffusion limitations within the enzyme particle. The increase in activity of lipase-catalysed acrylate synthesis in different supercritical fluids (SF_6, propane, ethane, ethylene, HCF_3 and CO_2) under the same conditions (45 °C, 11 MPa) was also associated with a decrease in fluid density.[24a] However, it is necessary to point out that, in all the assayed conditions, the use of M-SILP for enzyme immobilisation clearly improved productivity compared with CALB supported on silica-C_4. Thus, at 80 °C and 100 MPa, the productivity of M-SILP-**8**-CALB was 4.5- and 7-times higher than that obtained for M-SILP-**12**-CALB and silica-C_4 derivatives, respectively, reaching a total turnover number (TON) of 35.8×10^4 mol product/mol enzyme. Furthermore, all derivatives showed excellent operational behaviour, with practically no loss of activity during the assayed time, except in the case of the process carried out at 100 °C. The reaction was also very selective as the enzyme-catalysed reaction was fully directed towards the synthesis of **3**, due to the absence of free water molecules in the reaction system during the operation time. Only during the initial stages was the presence of the product of hydrolysis **4** observed for all derivatives (see Table 2). The yield of this product was extremely low and was not observed afterwards, probably because all the free residual water molecules remaining in the support after the immobilisation process were consumed.[8,11]

Different control experiments showed, first, that neither M-SILP-**8** nor M-SILP-**12** monoliths led to **3**

Table 2. Experimental assayed conditions and product yields obtained for M-SILP-**8**-CALB-catalysed synthesis of **3** in $scCO_2$.

Entry	Temp. [°C]	Pressure [MPa]	CO_2 density [g/mL]	Inlet mass flow [μmol/min]	Operation time [h]	Yield [%] **3**	**4**
A	40	10	0.627	10	10	55.7 ± 2.6	1.6 ± 0.2
B	60	10	0.301	20	10	86.2 ± 2.0	< 0.1
C	80	10	0.226	40	10	92.8 ± 3.2	< 0.1
D	100	10	0.191	20	5	24.3 ± 3.8	< 0.1

in the absence of CALB and, second, that no enzyme leaching occurred, as demonstrated by the lack of activity at the exit of the system. In fact, it was necessary to wash each monolith-CALB derivative with 100 mL of an aqueous solution of acetonitrile (50% v/v) to totally eliminate the adsorbed enzyme molecules. This revealed the possibility of washing out the deactivated enzyme and reactivating the reactor with fresh enzyme using simple protocols.[26]

These results should be analysed as a function of both the mass transport of the substrates to the active sites, and in the light of the different ability of each support to provide the enzyme with an appropriate microenvironment for catalysis. For example, the large pore distribution of monoliths (e.g., see Figure 3A for M-SILP-**8**), together with the high efficiency of $scCO_2$ to transport hydrophobic compounds, ensures very fast mass transfer directly through the pores.[7,23,25] On the other hand, ILs have been described as liquid immobilisation supports because multipoint enzyme-IL interactions (ionic, hydrogen bonds, van der Waals, etc) may occur, resulting in a supramolecular network able to maintain the protein conformation active.[8,11,12] In this case, monoliths containing covalently attached ILs not only provide an adequate microenvironment for lipase action, but also improve the mass-transfer phenomena of hydrophobic substrates and products from the $scCO_2$ phase, leading to a highly selective and stable immobilised enzyme even at high temperatures and pressures. The higher hydrophilicity and, probably, the different morphologies leading to higher back pressures in the case of M-SILP-**12** resulted in a clear decrease in the CALB's synthetic activity. Similarly, a clearly hydrophilic support, like silica gel, displayed the worst activity level for the whole temperature range under study.

Conclusions

We have developed new monoliths-supported ionic liquid phases (M-SILPs) with covalently attached IL-like moieties that transfer the desired properties of the liquid to the solid polymer. These M-SILPs can be used as highly efficient supports for the non-covalent immobilisation of enzymes and the preparation of bioreactors for lipase-catalysed ester synthesis in $scCO_2$. The efficiency of the system was dependent on both the microenvironment provided by the support and the supercritical conditions, which are involved in the mass-transfer phenomena. The methodology here reported represents a simple and straightforward strategy to improve the efficiency of an enzymatic reaction under non-classical conditions, allowing the development of green synthetic chemical processes.

Experimental Section

Safety Warning: Experiments using large amounts of compressed gases such as supercritical fluids are potentially hazardous and must only be carried out using appropriate equipment and safety precautions.

Materials

Candida antarctica lipase B (Novozym 525 L, EC 3.1.1.3) was from Novozymes S.A. (DK). The enzyme solution was ultrafiltered to eliminate all the low molecular weight additives, as follows: 25 mL Novozym 525 solution were diluted in 225 mL water, and the resulting solution was concentrated 10-fold by ultrafiltration at 4 °C, using a Minitan (Millipore) system equipped with polysulfone membranes (5,000 Da cut-off). This process was repeated 3 times, resulting in a CALB solution of 13.26 mg/mL, as determined by the Lowry method, and a purification degree of 1.6-fold. Modified silica gel (32–63 μm particle size, 6 nm pore diameter), containing butyl (Si-C_4) groups was obtained from Applied Separations Inc (Allentown, PA, USA). Molecular sieve UOP Type 4 (pore diameter 0.4 nm), substrates, solvents and other chemicals were purchased from Sigma–Aldrich-Fluka Co (Madrid, Spain), and were of the highest purity available.

Preparation of Monoliths

Monoliths **8** or **12** were molded into a stainless-steel cartridge (13 × 6.2 mm) of the ISCO 220SX high-pressure extraction apparatus. M-7 was prepared using a solution of 0.01 g of azobisisobutyronitrile (AIBN) in *p*-chloromethylstyrene (0.4 g), divinylbenzene (80% w/w, 0.6 g), toluene (0.58 g), and 1-dodecanol (1.75 g). The polymerisation mixture was stirred and purged with nitrogen for 3 min and poured into a mold. The stainless steel tubular mold was sealed at the two ends, and placed in a vertical position in a water bath. The polymerisation was allowed to proceed for 24 h at 70 °C. The seals were then removed, the tube was provided with fittings, attached to a high pressure pump, and THF was pumped through the column at a flow rate of 1 mL min^{-1} to remove the porogenic solvents and any other soluble compounds.

Polymer M-12 was prepared with similar preparation protocols using the polymerisation mixture show in Table 1.

Enzyme Immobilisation

Immobilised enzyme derivatives were prepared by simple adsorption of an aqueous solution of CALB (2 mg dissolved in 100 μL water) into monoliths, or silica-C4 (200 mg, previously packed in a stainless-steel cartridge). Then, each wet support was stored under controlled Aw (0.11) conditions over LiCl in a desiccator for 48 h at room temperature prior to use.

Drying of Substrates

Water was removed from **1** and **2** by adding molecular sieves (0.1 g/mL), shaking the resulting mixture for 24 h at room temperature, and finally storing them in the presence of the adsorbent.

Synthesis of 3 in $scCO_2$

A cartridge containig CALB-**8**, CALB-**12** or CALB-Si-C_4 derivative was placed into the ISCO 220SX system. The synthesis of **3** was carried out by continuous pumping of a substrate solution (1 M of **1** and **2**, respectively, in hexane) at 0.01–0.04 mL/min with an HPLC pump, and mixed with the $scCO_2$ flow of the system at 40–100 °C and 10 MPa (see Figure 1). The reactor was continuously operated for 5 h/d, followed by 19 h/d of storage into the ISCO system at room temperature. The reaction mixture was recovered by continuous depressurising through a calibrated heated restrictor (1 mL/min, 40 °C) for 30 min steps. Samples were analysed by GC. In all cases, the mass-balances of substrates and products from the outlet were consistent with the substrates mass-flow inlet.

GC Analysis

Analyses were performed with a Shimadzu GC-2010 instrument equipped with FID detector. Samples were analysed by a Beta DEX-225 column (30 m × 0.25 mm × 0.25 μm, Supelco), using butyl butyrate as internal standard and the following conditions: carrier gas (He) at 107 kPa (70 mL/min total flow); temperature program: 60 °C, 10 °C/min, 180 °C; split ratio, 50:1; detector, 300 °C.

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