Enzymes in neoteric solvents: From one-phase to multiphase systems

Pedro Lozano*

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Biphasic systems based on neoteric solvents, *e.g.* ionic liquids (ILs), supercritical carbon dioxide ($scCO_2$), fluorous solvents (FSs) and liquid polymers (LPs), represent interesting alternatives to organic solvents for designing continuous clean biotransformations methods in non-aqueous environments that directly provide pure products. The classical advantages of $scCO_2$ – its ability to extract, dissolve and transport chemicals – are complemented by the high catalytic efficiency of enzymes in liquid neoteric solvents (*e.g.* ILs, LPs or FSs). Enzyme behaviour in $scCO_2$ and ILs, as well as the phase behaviour of ILs/$scCO_2$, are key parameters for carrying out integral green bioprocesses in continuous operation. Experimental approaches, reactor designs and results are discussed in this Critical review.

1 Introduction

Over the past two centuries, the continuous growth of our knowledge of chemistry and the industrial application of the same has helped satisfy many of the critical needs of our society (foods, medicines, materials, *etc.*). Such advances have frequently involved the use of organic solvents, auxiliary materials used in chemical synthesis, where they act as media for mass-transport, reaction and product separation.[1] They are responsible for a large part of the environmental problems of processes in the chemical industry and have a great impact on cost, safety and health. The search for new environmentally benign non-aqueous solvents, which can easily be recovered/recycled, as well acting as efficient catalysts, is a priority for the development of green/sustainable chemical processes.[2] Four different non-aqueous neoteric solvents are the main targets of current academic and industrial research for applied biocatalysis: ionic liquids (ILs), supercritical fluids (SCFs), fluorous solvents (FSs) and liquid polymers (LPs) (see Fig. 1).

Departamento de Bioquímica y Biología Molecular B e Inmunología. Facultad de Química, Universidad de Murcia, P.O. Box 4021, E-30100, Murcia, Spain. E-mail: plozanor@um.es; Fax: +34 868 884148; Tel: +34 868 887392

Pedro Lozano

Pedro Lozano was born in Ceutí, Spain, in 1961. He received his PhD in Sciences (Chemistry) from the University of Murcia in 1988, under the supervision of Professors José L. Iborra and Arturo Manjón. Between, 1990 and 1991, he spent two years at the Centre de Bioingénierie Gilbert Durand, Toulouse (France) as a post-doctoral fellow under the supervision of Professor Didier Combes. In 1993, he returned to the Faculty of Chemistry at the University of Murcia as Assistant Professor in Biochemistry and Molecular Biology, being promoted to Full Professor in 2004. Since 1996, he has been Vice-Dean of the Faculty of Chemistry and coordinator of the Biochemistry Degree. His research activity has always been related with enzyme technology, his particular research interest focusing on the use of enzymes in ionic liquids and supercritical fluids.

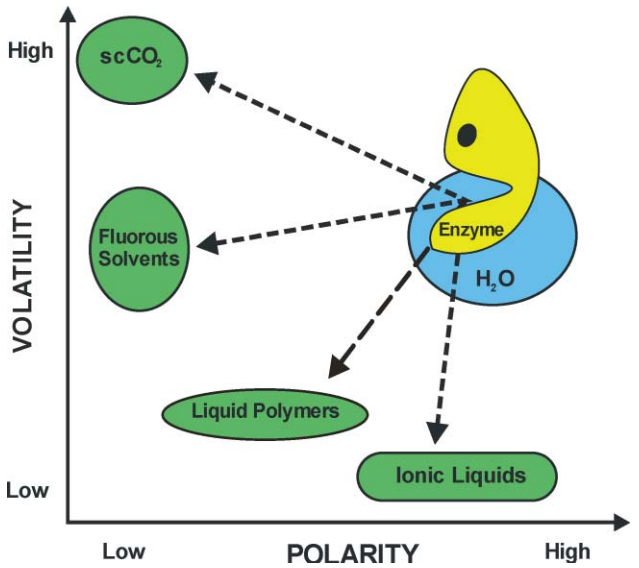

Fig. 1 Scheme of typical volatility and polarity characteristics of non-aqueous neoteric solvents suitable for enzyme catalysis.[2]

ILs are a new class of liquid solvent, and their use has led to a green chemical revolution because of their unique array of physico-chemical properties, which makes them suitable for numerous industrial applications. They are salts, and therefore entirely composed of ions, which are liquid below 100 °C or even close to room temperature. Typical ILs are based on organic cations (*e.g.* 1,3-dialkylimidazolium, tetraalkylammonium) paired with a variety of anions that have a strongly delocalized negative charge (*e.g.* BF_4, PF_6, bis((trifluoromethyl)sulfonyl)imide or NTf_2, *etc.*), resulting in

colourless easily handled materials of low viscosity.[3,4] Among the solvent properties of these fused salts, the miscibility/non-miscibility of ILs with molecular solvents, which can be tuned by selecting the appropriate cation and anion, is probably the most interesting feature. Since 2000, ILs have emerged as exceptionally interesting non-aqueous reaction media for biotransformations because of their exceptional ability to maintain enzymes in active and stable conformations.[5] The polar character of ILs contrasts with the non-miscibility with water of some of them, which is an interesting property for enzymatic catalysis, because water molecules are essential for maintaining enzymes active in non-aqueous environments.[6] Additionally, ILs are non-miscible with most hydrophobic organic solvents, thus providing a non-aqueous polar alternative for two-phase systems that has been widely applied to extracting products from reaction mixtures.

An SCF is defined as a state of matter at a pressure and temperature higher than its critical point, but below the pressure required to condense it into a solid. The possibility to manipulate the physical properties of these solvents by simply changing the pressure or temperature is unique to supercritical systems, which show exceptional abilities for extraction, reaction, fractionation and analysis processes.[7] As regards biocatalysis, the gas-like diffusivities and low viscosities of SCFs enhance the mass-transport rates of reactants to the active site of the enzymes and, since biocatalyst are insoluble in all SCFs, their recovery and reuse is straightforward.[8] A few fluids (CO_2, ethane, propane, butane, SF_6, CHF_3, *etc.*) have been assessed under supercritical conditions for their capacity to support enzyme catalysis because of the tendency of proteins to denature at high temperatures. Furthermore, as SCFs are low polarity solvents that preferentially dissolve hydrophobic compounds, they have been applied for processing these kinds of compound. The most popular SCF in enzyme catalysis is $scCO_2$, because it is chemically inert, non-toxic, non-flammable, cheap and readily available; it also exhibits relatively low critical parameters (*e.g.* $P_c = 73.8$ bar; $T_c = 31.0\,^{\circ}C$) that are suitable for biocatalysis, and is considered as a green solvent. Other SCFs are less attractive because of their flammability (*e.g.* ethane, propane), high cost (*e.g.* CHF_3) or poor solvating power (*e.g.* SF_6), and have therefore been used in few research studies.

Fluorous solvents (FSs), *e.g.* perfluoro-substituted alkanes, dialkyl ethers and trialkylamines, are another class of non-polar, hydrophobic, chemically inert, easily recyclable, and non-toxic solvents, with a higher density than corresponding non-fluorinated solvents.[8b,9] From the synthetic chemistry viewpoint, their temperature-dependent miscibility with classical organic solvents is a unique property, which makes them ideal for developing non-aqueous biphasic systems. Due to the differences in solubility between substrates and products, such biphasic fluorous/organic solvent mixtures can be used to turn a heterogeneous reaction medium into a homogeneous one with improved mass-transfer rates, and then reconvert it into a heterogeneous medium to facilitate product separation.

Liquid polymers (LPs), such as poly(ethyleneglycol) (PEG), poly(propylene glycol) (PPG) or poly(tetrahydrofuran) (PTHF), are another type of nonvolatile solvents that could be used in biocatalytic processes. As an alternative to ILs, Jessop's group demonstrated that the combination of PEG and $scCO_2$ constitutes a cheap and benign biphasic solvent system in homogeneous transition metals.[10a,b] However, because LPs are less polar than ILs, the polymers should be seen as complementary to, rather than replacements of, ILs. The LPs are tunable over a wide range of polarities by modification of the repeating unit and by expansion of the polymer with dissolved CO_2. PEG has a reasonable claim to the label "green solvent" because it is nonvolatile, nonflammable, nontoxic to humans, animals and aquatic life, and biodegradable by bacteria in soil and sewage.[10c]

However, the goal of green chemistry is much more than simply replacing hazardous solvents with environmentally benign ones. The selectivity of catalyzed processes is just as important, because of the importance of avoiding undesired reactions and/or by-products, and the need to facilitate product recovery. In this respect, enzymes clearly constitute the most powerful green tools for catalyzing chemical processes, since their activity and selectivity (stereo-, chemo- and regioselectivity) are far-ranging.[6] However, switching from natural aqueous media to non-aqueous solvents as the reaction medium for enzyme-catalyzed reactions is not always a simple alternative, because the native structure of the enzyme can easily be destroyed, resulting in deactivation. Water is the key component in all non-conventional media, because of the importance that enzyme–water interactions have in maintaining the active conformation of enzymes.[6,11]

Dry proteins are completely inactive, and a critical amount of water is required for them to function. It has recently been reported that proteins only achieve full biological activity when the surrounding water has approximately the same mass as the protein in question.[12] Thus, hydrophobic solvents typically afford higher enzymatic activity than hydrophilic ones, because the latter have a tendency to strip some of these essential water molecules from the enzyme molecules.[6] Water activity (A_w) has been proposed as a key parameter for determining the correct degree of hydration of enzymes in non-aqueous solvents.[13] Usually, enzymes fold by placing the non-polar residues into a hydrophobic core, while polar residues tend to move to the hydrated surface. A "memory" phenomenon is observed when an enzyme is placed in a dry hydrophobic system, because the biocatalyst is trapped in the native state as a consequence of the low dielectric constant of the medium. This intensifies intramolecular electrostatic interactions and enables the catalytic activity to be maintained.[6,14]

In this context, ILs, SCFs, FSs and LPs have received most attention worldwide for use as non-aqueous green solvents in enzyme catalysis, because of the improvements obtained in their catalytic properties and product recovery.[15] Additionally, biphasic catalytic systems based on a combination of these solvents with enzymes may represent the most important "arsenal" of green tools for developing integrally clean chemical processes of industrial interest in the near future.

2 Enzymes in ionic liquids

Biocatalysis in ILs has produced remarkable results under nearly anhydrous conditions. A large number of enzymes (*e.g.* lipases, proteases, peroxidases, dehydrogenases, glycosidases) and reactions (*e.g.* esterification, kinetic resolution, reductions, oxidations hydrolysis, *etc.*) have been tested in monophasic liquid systems based on ILs, due to their great ability to dissolve

Table 1 Some recent examples of lipase-catalyzed synthetic reactions in ILs at low water content[a]

IL	Reaction	Ref.
[Bmim][PF_6]	Synthesis of (*S*)-1-halo-2-octanol	17a
	KR of *rac*-menthol	17b
	Synthesis of acetamides	17c
	Synthesis of acyl L-canitine	17d
[$MEBu_3P$][NTf_2]	KR of 4-phenylbut-3-en-2-ol	17e
[Bmim][PF_6], [Bmim][NTf_2]	Polymerization of L-lactide	17f
[Bmim][BF_4]	Synthesis of ascorbyl oleate	17g
[Bmim][PF_6], [Emim][NTf_2], [Bmim][BF_4]	KR of *rac*-1-phenylethanol	17h
[Bmim][PF_6], [Bmim][BF_4]	KR of *rac*-3-phenyllactic acid	17i
[Bmim][PF_6], [Emim][NTf_2], [Emim][BF_4]	KR of aromatic *sec*-alcohols	17j
[CPMA][MS]	Synthesis of aliphatic esters	17k

[a] [Emim]: 1-Ethyl-3-methylimidazolium; [Bmim]: 1-Butyl-3-methylimidazolium; [Omim]: 1-Octyl-3-methylimidazolium; [$MEBu_3P$]: 2-Methoxyethyl-(tri-n-butyl)phosphonium; [CPMA]: cocosalkyl pentaethoxy methyl ammonium; [MS]: Methosulfate; [NTf_2]: Bis((trifluoromethyl)sulfonyl)imide; KR: kinetic resolution.

both polar and non-polar compounds.[5] While most water-miscible ILs have been shown to act as enzyme-deactivating agents at low water content, all the assayed water-immiscible ILs (*e.g.* [Bmim][NTf_2], [Bmim][PF_6], *etc.*) act as suitable reaction media for enzymatic catalysis in the same conditions. Additionally, the hygroscopic character of water-immiscible ILs (*e.g.* [Bmim][NTf_2] is able to absorb up 1.4% w/w water[16]) could be regarded as an additional advantage of these solvents, because enzymes require a certain degree of hydration to become actives. The direct addition of enzymes (*e.g.* aqueous solution, lyophilized powder of free enzyme, enzyme immobilized onto solid supports, or cross-linked aggregates) in the IL medium containing substrates is the usual approach applied for biotransformations in ILs (Fig. 2A). The reaction occurs at the selected temperature as a consequence of the activity and enantioselectivity displayed by enzymes. Lipases are by far the most widely used biocatalysts in ILs at low water content, and as such they are used for the synthesis of aliphatic and aromatic esters, chiral esters by kinetic resolution of racemic alcohols, carbohydrate esters, polymers, *etc*.[5] Table 1 shows some recent examples of lipase-catalyzed synthetic reactions in ILs at very low water content.

In many cases, the use of ILs improves the activity (*e.g.* synthesis of aliphatic esters,[17k] synthesis of acyl L-canitine,[17d] synthesis of ascorbyl oleate[17f]) or the selectivity (*e.g.* KR of *rac*-menthol,[17b] KR of *rac*-3-phenyllactic acid [17i]) of the enzyme with respect to that observed in organic solvents, although there seem to be no rules for predicting the outcome. In one example, the activity and enantioselectivity of lipase from *Bacillus cepacia* (BCL), in its free form, immobilized in a sol–gel (BCLxero), and as a CLEA, was tested for the acylation of different *sec*-alcohols in dry organic solvents, ILs and their mixtures. BCL–CLEA displays higher activity than BCLxero for all substrates in the ILs but loses its activity rapidly. BCLxero was suitable for kinetic resolution in the assayed ILs but the authors have found it impossible to label one IL as being better than another without taking the nature of the substrate into account.[17j] In another example, several novel phosphonium ILs were designed to evaluate the activity and enantioselectivity of the lipase-catalyzed kinetic resolution of 4-phenylbut-3-en-2-ol. The best results were obtained for the IL [$MEBu_3P$][NTf_2], which represents the first example where this reaction proceeded faster in an IL than in a conventional organic solvent, such as diisopropyl ether. However, when the 5-phenylpent-1-en-3-ol was used as substrate, an unexpected decrease in enantioselectivity was observed, which was attributed to a dependence of the lipase activity on the cationic part of the IL.[17e]

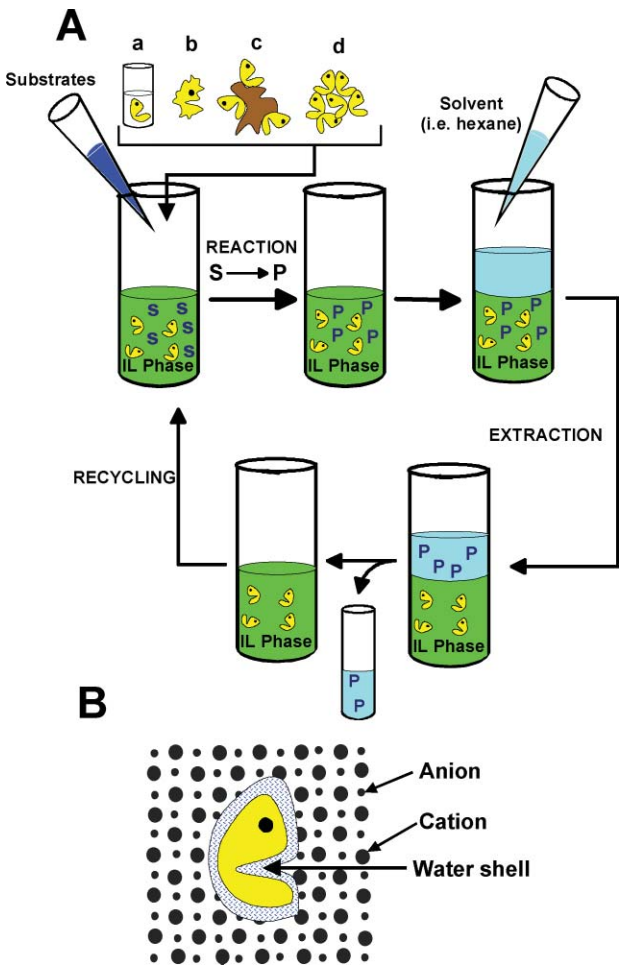

Fig. 2 **A**. Strategy for enzyme-catalyzed reactions in monophasic IL systems, including recycling and product separation: (a) enzyme in aqueous solution; (b) lyophilized free enzyme; (c) immobilized enzyme on solid supports; (d) cross-linked enzyme aggregates. **B**. Representation of enzyme molecule in water-immiscible ILs.[19]

Further highlighting the suitability of ILs as reaction media for biocatalysis, the excellent stability displayed by enzymes in water-immiscible ILs should be mentioned.[18] The maintenance of the native conformation of proteins in these media has been demonstrated by spectroscopic techniques (*e.g.* fluorescence, circular dichroism, FT-IR, *etc.*),[19] underlining the excellence of water-immiscible ILs to protect enzymes against the usual unfolding that occurs in non-aqueous environments (*e.g.* the half-life time of CALB in [Btma][NTf_2] at 50 °C is 1660 times higher than that in hexane).[19d] The structure of ILs has been described as an extended network of cations and anions connected by hydrogen bonds, where the incorporation of other molecules in the IL net may give rise to the formation of polar and non-polar regions. Thus, wet ILs are nano-structured materials which allow neutral molecules to reside in less polar regions and ionic or polar species to undergo faster diffusion in the more polar or wet regions.[20] In this context, the observed stabilization of enzymes in water-immiscible ILs could be attributed to their inclusion in hydrophilic gaps of the network, where they are surrounded by a strong ionic net (Fig. 2B). The extremely ordered supramolecular structure of ILs in liquid phase might be able to act as a "mould", maintaining the active 3-D structure of the enzyme in aqueous nano-environments that avoid unfolding. Free enzymes suspended in IL systems can be considered as carrier-free immobilized enzymes. For example, enzyme–IL systems can be reused in consecutive operation cycles, because enzymes cannot be separated from ILs by liquid–liquid extraction (*e.g.* with buffer or aqueous solutions), since it is necessary to filter the enzyme–IL solution through ultrafiltration membranes to clean the IL.[18,19]

Despite of all the excellent catalytic properties shown by enzymes in IL systems, the recovery of products is also a key step in developing any integral clean chemical process. Since many molecular solvents (*e.g.* water, hexane, toluene) are immiscible with ILs, biphasic systems based on IL–water or IL–organic solvent have been assayed for biocatalytic processes.[21] As an example, the kinetic resolution of *rac*-ibuprofen has been carried out in a water–IL biphasic medium.[22a] The system was operated by coupling two lipase reactions (esterification and hydrolysis, respectively) with a membrane containing supported ILs (Fig. 3A). As the IL-based supported liquid membranes permit the selective transport of organic molecules, the system provides for the easy and selective permeation of the synthesized (*S*)-ibuprofen ester through the membrane.[22b] The ester is then hydrolyzed by another lipase that provides a successful resolution of the racemic mixture. In another example, for the Novozym-catalyzed synthesis of 6-*O*-alkanoyl-methyl-D-glucopyranosides by esterification of methyl-D-glucopyranoside with fatty acids in biphasic systems, coating the immobilized lipase with small amounts of ILs (*i.e.* 1-butyl-4-methylpyridine hexafluorophosphate) provided superior efficiency (80%) and broader substrate tolerance than solvent-free transformation, due to the greater polarity of the ionic liquids used as a liquid-film coating.[23]

However, in the case of hydrophobic compounds, liquid–liquid extraction with organic solvents is the most commonly used strategy for recovering the products obtained by the enzyme acting in ILs,[5] although the use of these solvents must be regarded as a clear breakdown as regards the greenness of

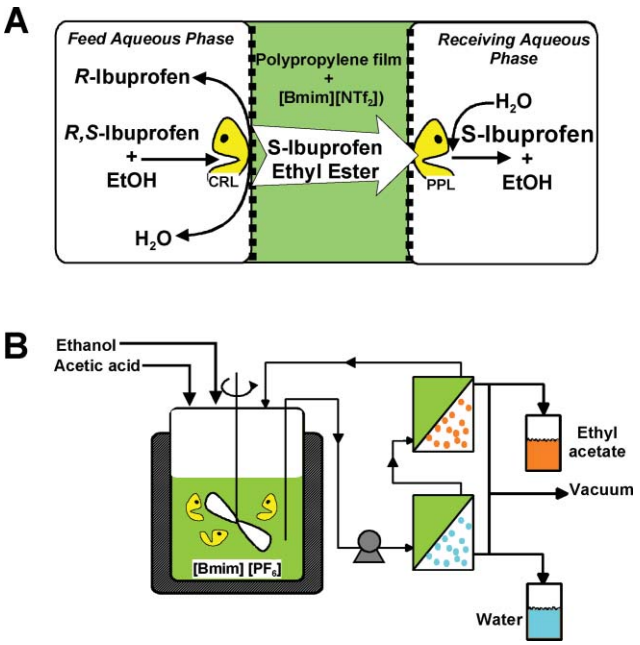

Fig. 3 **A.** Facilitated enantioselective transport of (*S*)-ibuprofen through a supported IL membrane with immobilized lipase. CRL, *Candida rugosa* lipase; PPL, porcine pancreas lipase.[22a] **B.** Continuous membrane reactor for the enzymatic synthesis of ethyl acetate in [Bmim][PF_6].[24b]

the process. To overcome this problem, alternative strategies for product recovery based on membrane technology (*e.g.* pervaporation) have been applied.[24] Pervaporation processes occur under vacuum to allow the evaporation of a selective component from a mixture after permeation through a membrane, where the driving force for the separation is the difference in the partial pressures of the components on the two sides of the membrane. For example, the immobilized CALB-catalyzed esterification of acetic acid and ethanol in [Bmim][PF_6] was carried out in a membrane reactor able to remove both the ethyl acetate and water produced by a double pervaporation system using hydrophobic and hydrophilic membranes, respectively, in continuous operation for 72 h without any loss in the enzyme activity (see Fig. 4B).[24b] However, product recovery from IL–enzyme reaction media by another non-aqueous green solvent, such as $scCO_2$, is nowadays considered the most interesting strategy for developing integral clean chemical processes.

3 Supercritical biocatalysis

Since 1985, the use of SCFs as non-aqueous reaction media for enzyme catalysis has been an active area of research.[8] In spite of enzymes (*e.g.* lipases, trypsin, chymotrypsin, penicillin acylase, cholesterol oxidase, *etc*) being able to catalyze many chemical transformations (*e.g.* esterification, hydrolysis, alcoholysis, *etc.*), it has been demonstrated how the enzyme activity is widely affected by the nature of the SCF fluid. For example, the activity of lipase-catalyzed ethyl butyrate synthesis by esterification was higher in near-critical propane than in $scCO_2$, which was attributed to the capability of CO_2 to strip the essential water molecules from the enzyme microenvironment.[25a] Furthermore, the chemically inert character of CO_2 might be doubted as

Table 2 Some examples of synthetic products obtained by lipase action in $scCO_2$

Products	Reaction conditions	Yield (%)	Ref.
Ethyl butyrate	40 °C, 100 bar, 24 h	30	25a
(*R*)-2-Phenyl-1-propyl butyrate	40 °C, 100 bar	—	26c
(*R*)-1-Phenylethyl acetate	50 °C, 100 bar	44 (>97% ee)	26d
Ethyl oletate	40 °C, 150 bar, 3 h	95	27a
Butyl butyrate	50 °C, 90 bar, 3 h	100	27b
	50 °C, 100 bar, 5 h	80	27c
Chlorogenic esters	30–55 °C, 150–250 bar, 25 h	77–85	27d
Cocoa butter analog	40 °C, 100 bar, 3 h	30–75	27e
Fatty acid ethyl esters	30–70 °C, 50–150 bar	80	27f
(*R*)-Citronellyl oleate	31.1 °C, 84.1 bar	3.6 (>98.9% ee)	27g
Glucosyl palmitate	50 °C, 65 bar, 4 h	25	28b
(*R*)-1-Phenylethyl acetate	42 °C, 130 bar	48 (>99% ee)	30a
(*R*)-1-Phenylethyl acetate	50 °C, 200 bar, 6 h	48 (>99% ee)	30b
(*S*)-Glycidyl butyrate	35 °C, 140 bar, 10 h	30 (83% ee)	30c
(*S*)-Propyl ester of ibuprofen	50 °C, 100 bar, 23 h	75 (70% ee)	30d

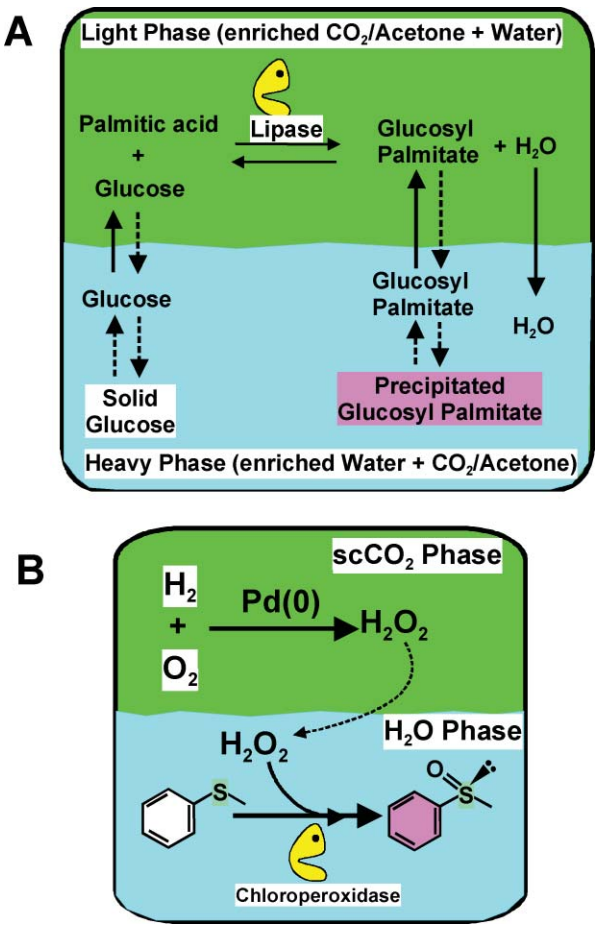

Fig. 4 **A.** Enzymatic esterification of glucose and palmitic acid in a high-pressure acetone–CO_2 system.[28b] **B.** Chemo-enzymatic sulfoxidation of thioanisole in $scCO_2$–H_2O biphasic media.[29b]

regards its interaction with proteins. CO_2 forms carbamates with ε-amino groups of lysine residues placed on the enzyme surface, and decreases the pH of the aqueous layer around the enzyme. In a recent example, the influence of temperature, exposure time and pressure-compressed CO_2 and propane on the secondary structure of horseradish peroxidise (HRP) has recently been reported using far-UV–circular dichroism analysis.[25b] This work shows how treating aqueous solutions of enzyme with propane did not induce changes in the secondary structure content of HRP, resulting in good stability. In contrast, incubation with CO_2 led to a significant loss of the HRP secondary structure, which was accompanied by a significant decrease in the enzyme activity. However, the solid commercial HRP showed no decrease in its activity after treatment with pressurized CO_2 or propane, although treatment in both solvents provoked a loss in the secondary structure. All these facts lend weight to the final sentence of the review published by Beckman and Russel in 1999, "*…the advantages of replacing conventional organic solvents with supercritical fluids have not been fully demonstrated yet*".[8a]

Since 2000, several strategies have been developed to protect enzymes against these adverse effects of $scCO_2$; for example, covalent attachment on supports coated with hydrophilic polymers,[26a] the coating of free enzymes with hydrophobic silicates by a sol–gel approach,[26b] the entrapment of enzymes in silica-aerogels[26c] and the use of cross-linking enzyme aggregates.[26d]

Lipases in $scCO_2$ are the most widely studied systems, because of the catalytic promiscuity of these enzymes towards hydrophobic substrates, and the excellent ability of this fluid to dissolve and transport hydrophobic compounds.[8] Thus, the synthesis of esters by esterification or transesterificaton (*e.g.* by alcoholysis, acidolysis or transesterification) is the most popular enzymatic process in $scCO_2$ (see Table 2), being applied to the modification of oils and fats, including the production of biodiesel.[27] In the case of hydrophilic compounds (*e.g.* sugars), multiphase catalytic strategies have been developed to overcome their low solubility in $scCO_2$ (*e.g.* adsorption of sugars in solid support,[28a] molecular solvent–$scCO_2$ biphasic systems,[28b] *etc.*). For example, the esterification of palmitic acid and glucose by Novozyme 435 was performed in $scCO_2$-expanded acetone. An amount of acetone up to 3% (v/v) is required to ensure that the reaction takes place in an expanded liquid phase at 50 °C and 65 bar. The presence of a small amount of water (up to 0.3% v/v) improved esterification performance, because it helped maintain the hydration level of the enzyme and the removal of water as a by-product, due to the multi-phase distribution of the acetone–CO_2–water–glucose system (See Fig. 4A).

The suitability of non-lipase enzymes (*e.g.* chloroperoxidase, glucose oxidase, *etc.*) to catalyze oxidation reactions in SCFs

has also been reported.[8,29] An interesting example is the chemo-enzymatic cascade oxidation in $scCO_2$–water biphasic media to catalyze the enantioselective sulfoxidation of thioanisole (see Fig. 4B).[29b] In this system, Pd(0) catalyses the formation of H_2O_2 from H_2 and O_2 in the supercritical phase; the peroxide is subsequently used by the chloroperoxidase as an oxidant for the asymmetric sulfoxidation in the aqueous phase. In spite of the moderate reaction yields (14–60%), and the significant activity loss of the enzyme with time (up to 90% in 3 d at 40 °C and 130 bar), this work exemplifies the potential of compartmentalisation of the catalytic processes in multiphase systems.

The asymmetric synthesis of esters is probably the most active area of research, where the excellent enantioselectivity shown by several lipases (*e.g.* CALB), combined with the unique properties of $scCO_2$, have successfully permitted the chiral resolution of a large number of racemates (1-phenylethanol, glycidol, ibuprofen, *etc.*).[30]

An important feature in supercritical biocatalysis is reactor design, which should facilitate the control of mass-transfer limitations, reaction conditions (pressure and temperature) and product recovery. Many types of reactors *e.g.* stirred-tank, packed-bed, see Fig. 5) have been used to carry out biotransformation in continuous or discontinuous operation.[25-29] In all cases, the reactors are made of stainless steel and contain a variety of control devices. In some cases, the supercritical reactor also contains elements to simultaneously perform both the biocatalytic and product separation steps (*i.e.* by producing a back-pressure cascade in a series of coupled high-pressure separator vessels,[27a] or by using a ceramic membrane reactor[27b]).

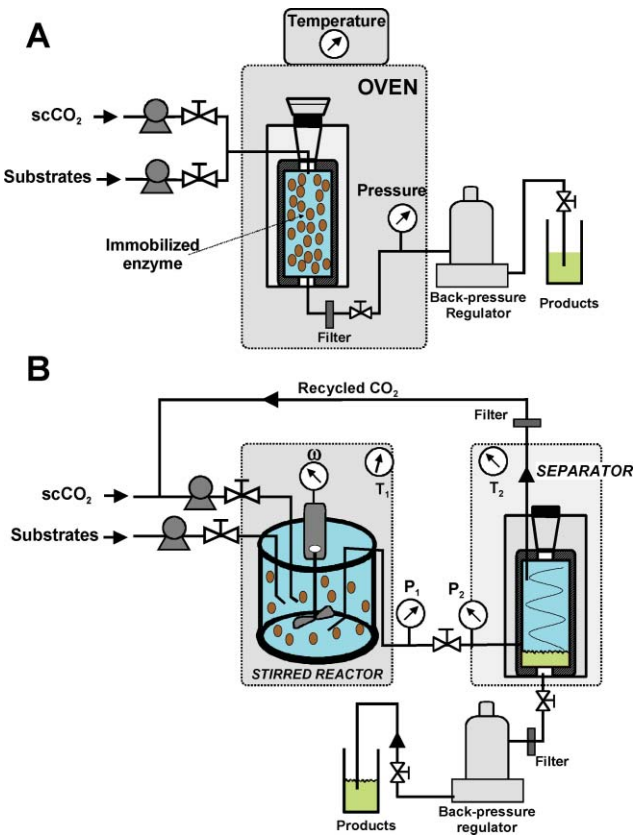

Fig. 5 **A**. High-pressure packed-bed enzymatic reactor for biotransformation in $scCO_2$. **B**. High-pressure stirred-tank reactor coupled to a separator vessel for enzyme-catalyzed transformations, product recovery and reuse of $scCO_2$.

Pressure, temperature and water content are the most important environmental factors affecting enzymatic catalysis in SCFs, particularly their activity, enantioselectivity and stability.[31] In addition to the specific effect of CO_2 on proteins, high pressure may also have a negative impact on enzyme conformation. The rapid release of CO_2 dissolved in the bound water of the enzyme during depressurization has also been claimed to produce structural changes in the enzyme and to cause its inactivation.[32] However, through changes in pressure in the vicinity of the critical points (*e.g.* from 77 to 90 bar), a clear improvement in lipase-catalyzed ester synthesis has been observed. For example, in the synthesis of butyl butyrate catalyzed by CALB, the synthetic activity was exponentially increased by the decrease in $scCO_2$ density accompanying different combinations of pressure and temperature.[27b] In another example, the lipase-catalyzed esterification of *rac*-citronellol with oleic acid in $scCO_2$ an ester product with an >99.9% ee can be obtained simply by manipulating pressure and temperature around the critical point.[27g] Conversely, for the same immobilized lipase-catalyzed continuous kinetic resolution of *rac*-1-phenylethanol, it was demonstrated that changes in pressure did not greatly affect conversion or *E*-values, even when the pressure was increased to 500 bar.[30a]

Temperature affects enzyme activity much more than pressure because of enzyme deactivation processes.[8] The optimal temperature of enzymatic processes in SCFs is related with pressure because both parameters control solvent properties. The negative effect of temperature on enzymes in supercritical conditions has been related to changes in the hydration level of the enzyme.

Water concentration is a key factor in supercritical biocatalysis because of its great influence on enzyme activity and stability.[31] It should be noted that $scCO_2$ may dissolve up to 0.3–0.5% (w/w) water, depending on the temperature and pressure, and lead to enzyme deactivation through dehydration in continuous operation. On the other hand, if the water content in the supercritical medium is too high or if the water molecules are produced in the reaction, the increased humidity may also lead to enzyme deactivation. The appropriate selection of a support for the immobilized enzyme, or the addition of salt hydrates (*e.g.* $Na_4P_2O_7{\cdot}10H_2O$), has been successfully applied to preserve the essential water layer around the enzyme.[26]

In spite of the advantages obtained with all these experimental approaches to preserve the catalytic properties of enzymes, the best results for supercritical biocatalysis were observed when the enzyme was applied in suspension or coated with other non-aqueous green solvents, such as ILs.

4 Multiphase biocatalytic systems based on ILs and $scCO_2$

Compared to heterogeneous catalysis, homogeneous catalysis has the drawback of making catalyst recycling and product separation both costly and difficult. In contrast, heterogeneous catalysis in multiphase operation offers promising opportunities

for developing industrial processes (*e.g.* the catalyst operates in one phase and the product is extracted in the second phase).[33] To create a cleaner and more sustainable chemistry, we must attempt to reduce or even eliminate the use of VOCs in all catalytic systems. As stated above, the recovery of solutes dissolved in ILs represents a clear breakdown in the greenness of any such process if VOCs are used to extract the same in liquid–liquid biphasic systems. The pioneering work of Brennecke's group in 1999 showed that ILs (*e.g.* [Bmim][PF_6]) and $scCO_2$ form biphasic systems. Additionally, although $scCO_2$ is highly soluble in the IL phase and is able to extract previously dissolved hydrophobic compounds (*e.g.* naphthalene), the same IL is not measurably soluble in the $scCO_2$ phase.[34] This discovery was crucial for further developments in non-aqueous multiphase green biocatalytic processes involving both biotransformation and extraction steps.

The use of enzymes in multiphase systems based on ILs and $scCO_2$ as reaction media for biocatalysis was originally described in 2002, and represented the first operational approach for the development of fully green chemical processes in non-aqueous environments.[35] Using this approach, the $scCO_2$ flow can serve both to transport the substrate to the IL phase containing the biocatalyst, and to extract the product(s) from the IL. In this way, the product(s) obtained by decompression of the SCF are free from IL and from other organic solvent residues, whereas CO_2 can be recycled by re-compression. Additionally, if the reaction product does not require any further purification, the approach enhances the economic benefit of the process, because the system runs as a 'black box' able to transform pure substrate to pure products without waste generation.

4.1 IL–$scCO_2$ biphasic systems

The phase behaviour of IL–$scCO_2$ systems, including the partitioning behaviour of organic compounds between both solvents, has recently been reviewed.[36] Knowledge of this phase behaviour is essential for developing any process because it determines the contact conditions between $scCO_2$ and solute, as well as, the conditions for reducing the viscosity of the IL phase, thus enhancing the mass-transfer rate of any catalytic system.

Preliminary investigations into [Bmim][PF_6]–$scCO_2$ mixtures indicated that these systems behave as biphasic systems, the solubility of CO_2 in the IL phase increasing with pressure (up to 0.32 mole fraction at 93 bar and 40 °C), while temperature has a small effect on CO_2 solubility.[37a] Further studies on [Bmim][PF_6]–$scCO_2$ phase behaviour at high pressure (up to 970 bar) pointed to two distinct phases for all the conditions assessed.[37b] At high pressures, the density of the CO_2 phase increases but, since the IL phase does not expand, the two phases never become one phase. The water content of ILs has an important effect on these systems, as seen from the increased solubility of CO_2 when ILs are previously dried (*e.g.* the mole fraction of CO_2 in dry [Bmim][PF_6] is 0.54, whereas for the wet (water-saturated) IL sample it is only 0.13). A similar phase behaviour to [BMim][PF_6] was observed for other IL–$scCO_2$ systems (*e.g.* [Omim][PF_6], [Omim][BF_4], [Bmim][NO_3]), where the solubility of CO_2 in the IL-rich phase increased proportionally with the increase in the alkyl chain length of the cation, being highest for the ILs with fluorinated anions (*i.e.* [PF_6]). In general, the solubility of CO_2 in IL increases with increasing pressures but the exact amount of CO_2 dissolved in the IL phase varies significantly (*e.g.* at 70 bar, the solubility of CO_2 in [Emim][$EtSO_4$] was 0.36 mole fraction whereas, it was 0.63 in [Omim][PF_6]).[37b] In the same context, it was demonstrated how as CO_2 pressure is increases (up to 287 bar), the viscosity of several 1-alkyl-3-methyl-imidazolium [NTf_2] ILs dramatically decreases. Also, while the ambient pressure viscosity of ILs increases significantly with chain length, the viscosity of all the CO_2-saturated ILs becomes very similar at high CO_2 pressures (2–3 mPa s).[37c]

However, in spite of the low solubility of ILs in $scCO_2$ (*e.g.* 5×10^{-7} mole fraction of [Bmim][PF_6] in $scCO_2$ at 138 bar and 40 °C),[37a] the presence of other compounds (acetone, ethanol, *etc.*) may enhance the solubility of ILs in the $scCO_2$ phase as a result of the strong interaction of these co-solvents with the IL due to their strong polarity. The ability of these co-solvents to increase the solubility of ILs (*e.g.* [Bmim][PF_6], [Bmim][BF_4]) in $scCO_2$ closely reflects the increase in its dipole moment (*i.e.* acetonitrile > acetone > methanol > ethanol > hexane).[38]

The extraction of compounds dissolved in ILs with $scCO_2$ is probably the most attractive feature of these biphasic systems because both IL and CO_2 can be recycled and extraction does not involve cross-contamination, which represents a green approach for the recovery of solvent-free solutes.[2] The extraction of naphthalene from [Bmim][PF_6] by $scCO_2$ was the first example of solute recovery in green non-aqueous conditions, the process providing a product extract containing no detectable liquid solvent.[34] Further studies by Brennecke's group determined the solubility of twenty organic solutes containing different substituent groups (*e.g.* halogen, alcohol, amide, ester, ketone, *etc.*) in [Bmim][PF_6], and provided quantitative data on extraction recovery rates for these aromatic and aliphatic compounds from this IL with $scCO_2$. In this respect, it should be noted how compounds with a low solubility in the IL phase (*e.g.* benzene or chlorobenzene) required the least amount of CO_2 for recovery. Also, solutes with high dipole moment gave low distribution coefficient values (defined as the ratio between solute mole fractions in the $scCO_2$ and in the IL phases, respectively) because of their high affinity for IL and low affinity for CO_2, which makes extraction more difficult.[39]

Another important feature of $scCO_2$ which facilitates product separation from IL media is the ability of CO_2 to manipulate the phase behaviour in IL–organic or IL–water systems by increasing the pressure. Using a methanol solution in [Bmim][PF_6] as a model, it was demonstrated that pressurization with CO_2 induces the formation of three phases, two of them liquid (see Fig. 6).[40] At ambient temperature, methanol and [Bmim][PF_6] are fully miscible in all proportions, but it is possible to design an approach to separate ILs from organic compounds by using CO_2. The pressurization of the organic compound–IL solution (L) with CO_2 results in the formation of a second liquid phase. The densest liquid is rich in IL (L1), the next liquid phase is rich in the organic compound (L2), while the third vapour phase (V) is mostly CO_2 with some organic compounds. The pressure and temperature conditions in which the second liquid phase appears is called the lower critical endpoint (LCEP), which is dependent on the initial amounts of methanol and IL. In these conditions, the L2 phase expands significantly with increased

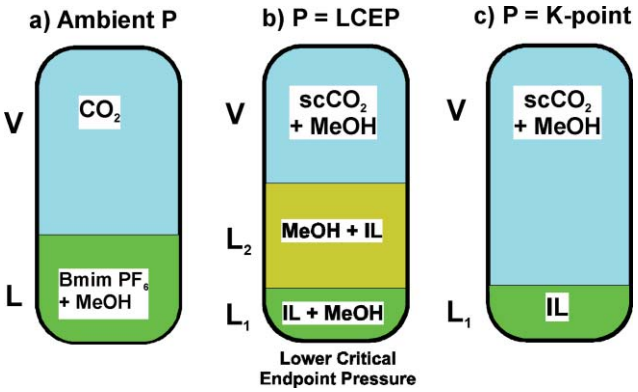

Fig. 6 Schematic representation of the influence of pressure on the phase behaviour of IL–methanol–CO_2 ternary systems, (a) ambient pressure (P); (b) lower critical endpoint pressure (LCEP); (c) K-point pressure. (L, L1 and L2: liquid phases; V: gas phase).[40]

CO_2 pressure, while the IL-rich phase (L1) expands relatively little. As the pressure of CO_2 increases again and reaches another critical point, called the K-point, the methanol-rich phase (L2) merges with the vapour phase (V), while the resulting $scCO_2$–organic compound phase contains no detectable IL. These "*phase equilibrium switches*" can be reversed by modifying the CO_2 pressure at a particular temperature and IL–methanol proportion and they are very interesting for both reaction and separation processes using ILs.[40]

The growing number of possible applications of IL–$scCO_2$ biphasic systems in synthesis and extraction processes was recently increased by the several phase equilibrium studies for compound–IL–$scCO_2$ systems with larger organic molecules (*i.e.* naphthalene,[41a,b] 1-phenylethanol,[41c,d] naproxen,[41e] *etc.*). For the case of naphthalene in a [Bmim][PF_6]–CO_2 system, it was found that its concentration in the CO_2-rich phase decreased with an increase in temperature (30–60 °C range), but increased with an increase in pressure (80–200 bar range).[41a] In the same way, the extraction of naphthalene from [Bmim][CF_3SO_3] using $scCO_2$ or sc-propane was also studied in continuous and batch processes. This IL showed a thermal stability higher than [Bmim][PF_6], while the ternary phase equilibria of CO_2–[Bmim][CF_3SO_3]–naphthalene and propane–[Bmim][CF_3SO_3]–naphthalene were determined in a wide pressure and molality of solute range at 50–150 °C, pointing to the suitability of these systems for long-time separation processes of high boiling point organics from ILs.[41c] In another example, the solubility of 1-phenylethanol in a [Bmim][BF_4]–$scCO_2$ system was also investigated at pressures up to 140 bar and a temperature range from 9 to 95 °C.[41d] The formation of hydrogen bonds between the hydroxyl group of 1-phenylethanol and the [BF_4] anion was attributed to lower CO_2 solubility, compared with a similar solute with a carbonyl-carbon, such as 1-(4-isobutylphenyl)ethanol, which does not form hydrogen bonds with the ionic liquid anion. In the same way, the solid–liquid and liquid–vapour equilibria of the ternary system (*S*)-naproxen–[Bmim][BF_4]–$scCO_2$ has been studied at 10–50 mol% CO_2 concentrations, 1–140 bar and 37–97 °C. This example shows how the (*S*)-naproxen is poorly soluble in [Bmim][BF_4], whereas pressurization with $scCO_2$ permits its complete dissolution. Therefore, the amount of dissolved CO_2 can be used to switch the miscibility of the solid solute in the system, allowing operational conditions to be selected for reactions (as one homogeneous phase), or for separations (such as heterogeneous solid + liquid phase).[41e] This approach has also be used to ascertain the anti-solvency behavior of $scCO_2$ as a way to recover methyl (*Z*)-α-acetamidocinnamate (MAAC) from [Bmim][BF_4] by crystallization. This work demonstrated how the MAAC can be recovered from the IL by either using a shift to higher CO_2 concentrations at constant temperature (anti-solvent crystallization) or by using a shift to lower temperatures at constant CO_2 concentration (thermal shift).[41f]

Further investigations into [Bmim][PF_6]–ethanol–water–CO_2 quaternary mixtures at 50 °C found a varied phase behaviour, ranging from total miscibility, through partial miscibility to nearly complete phase separation, which can also be useful in reaction–separation cycles.[42a] These water–IL–$scCO_2$ ternary mixtures have also recently been applied to separate uranyl ions $[UO_2]^{2+}$ from an aqueous nitric acid solution by extraction into $scCO_2$ phase using the [Bmim][NTf_2] containing tri-*n*-butyl phosphate (TBP) as a complexing agent. The TBP was able to complex the uranyl ions and to dissolve it into the IL phase, after which uranium is transferred from the IL to the supercritical phase, identified as a $UO_2(NO_3)_2$–$(TBP)_2$ complex. This approach has potential applications in the field of nuclear waste management for extracting other actinides.[42b]

Another interesting feature of IL–$scCO_2$ phase concerns the change in melting point of ILs, as demonstrated by Leitner's group.[43] They observed pronounced melting point depressions, some even exceeding 100 °C, induced by compressed CO_2 in some ILs based on ammonium or phosphonium cation. In the case of tetrabutylammonium tetrafluoroborate ([Bu_4N][BF_4], mp 156 °C), equilibration with high-pressure CO_2 at 150 bar resulted in a melting point depression of 120 °C. This CO_2-induced depression in the melting point may make available some new solvents for IL–$scCO_2$ biphasic catalysis, as well as facilitate IL recovery and reuse. Further studies of the same group used two different high-pressure devices to determine the solid–liquid–vapour boundaries (melting point depressions) and the composition of coexisting phases in 28 IL–$scCO_2$ systems. They observed CO_2-induced melting point depressions ranging from 7 °C to 128.8 °C, suggesting that the large depressions may result from weak Lewis acid–Lewis base interactions of the acidic carbon in CO_2 with the basic moieties of the IL.[43b]

All these features underline the advantages for using multiphase (bio)catalytic systems based on IL and $scCO_2$, for the development of sustainable chemical processes in non-aqueous media. Many recent examples in chemical catalysis (*e.g.* hydroformylation of 1-octene,[44a] polymerization of styrene,[44b] synthesis of tocopherol,[44c] hydrogenation of methyl-α-acetamido cinnamate,[44d] Friedel–Crafts acylation,[44e] *etc.*) and biocatalysis[45] (see next section) underline the exciting potential of these systems.

4.2 Biocatalytic processes in IL–$scCO_2$ biphasic systems

Enzymatic reactions based on ILs and $scCO_2$ are interesting alternatives to organic solvents for designing clean synthetic chemical processes that provide pure products directly. The classical advantages of $scCO_2$ to extract, dissolve and transport chemicals are tarnished in the case of enzymatic processes

Table 3 Biocatalytic processes in IL–$scCO_2$ biphasic systems[a]

Reaction	IL	Conditions	Ref.
Synthesis of butyl butyrate	[Emim][NTf_2], [Bmim][NTf_2]	125–150 bar, 40–100 °C	35a
KR of 1-phenylethanol	[Emim][NTf_2]	100–150 bar	35a
	[Bmim][NTf_2]	100–150 bar	35b
	[Btma][NTf_2]	100–150 bar	46a
	[Toma][NTf_2]	100–150 bar	47
KR of glycidol	[Emim][NTf_2]	100 bar, 50 °C	46c
	[Bmim][NTf_2]	100 bar, 50 °C	46c
	[Toma][NTf_2]	100 bar, 50 °C	46c
	[Bmim][PF_6]	100 bar, 50 °C	46c
Synthesis of several alkyl esters (*i.e.* butyl acetate)	[HOPrtma][NTf_2]	100 bar, 50 °C	46b
	[CNPrtma][NTf_2]	100 bar, 50 °C	46b
	[Btma][NTf_2]	100 bar, 50 °C	46b
	[CNPtma][NTf_2]	100 bar, 50 °C	46b
	[Htma][NTf_2]	100 bar, 50 °C	46b
Butanolysis of triolein	[Toma][TFA]	85 bar, 35 °C	48a
KR of 2-octanol	[Omim][PF_6]	110 bar, 35 °C	48b
	[Omim][NTf_2]	110 bar, 35 °C	48b
Synthesis of citronellyl laurate	[Bmim][PF_6]	100 bar, 60 °C	48c
	[Bmim][BF_4]	100 bar, 60 °C	48c
KR of 2-phenyl-1-propanol	[Bmim][PF_6]	100 bar, 35 °C	49
DKR of 1-phenylethanol	[Emim][NTf_2]	100 bar, 50 °C	53
	[Bmim][PF_6]	100 bar, 50 °C	53
	[Btma][NTf_2]	100 bar, 50 °C	53
Synthesis of citronellyl propionate	SILP	100 bar, 40–100 °C	55

[a] [HOPrtma]: (3-Hydroxypropyl)trimethylammonium; [CNPrtma]: (3-Cyanopropyl)trimethylammonium; [Btma]: Butyltrimethylammonium; [CNPtma]: (5-Cyanopentyl)trimethylammonium; [Htma]: Hexyltrimethylammoniuum; [TFA]: Trifluoroacetate; SILP: Supported ionic liquid phase. KR: Kinetic resolution; DKR: Dynamic kinetic resolution. See Table 1 for other abbreviations.

because of its denaturative effect on enzymes. On the other hand, ILs have shown themselves to be excellent non-aqueous environments for enzyme catalysis. The use of IL–$scCO_2$ biphasic systems as reaction media for enzyme catalysis has opened up new opportunities for the development of integral green processes in non-aqueous environments (See Table 3).[45]

In this context, a new concept for biphasic biocatalysis in non-aqueous environments, whereby a homogeneous enzyme solution is immobilized in the IL phase (catalytic phase), while substrates and products reside largely in the SCF phase (extractive phase), directly providing products, has been put forward as the first approach of integral green bioprocess in non-aqueous media (see Fig. 7). The system was tested for two different reactions catalyzed by CALB: the synthesis of aliphatic esters by transesterification from 1-alkanols and vinyl esters (*e.g.* butyl butyrate from vinyl butyrate and 1-butanol), and the kinetic resolution of *rac*-1-phenylethanol in a wide range of conditions (100–150 bar and 40–100 °C). In these conditions, the enzyme showed an exceptional level of activity, enantioselectivity (>99.9% ee) and operational stability (*e.g.* the enzyme only lost 15% activity after 11 cycles of 4 h).[35a] These excellent results obtained for biotransformations in $scCO_2$ using the enzyme coated with ILs were corroborated in extreme conditions of 100 bar and 150 °C.[46a]

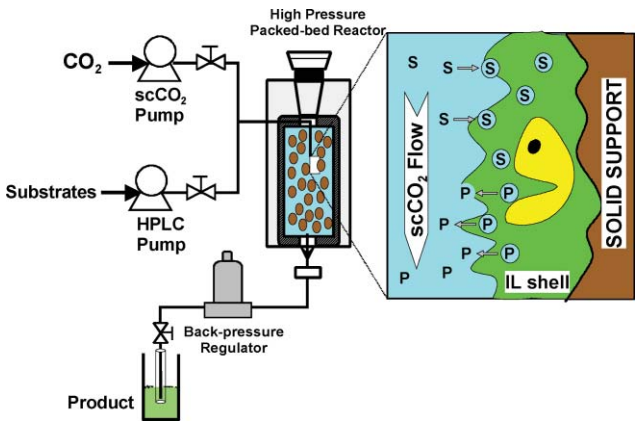

Fig. 7 Set-up of a continuous green enzymatic reactor working in an IL–$scCO_2$ biphasic system.[35a,46]

Further studies on these IL–$scCO_2$ biocatalytic systems attempted to throw light on the role of mass-transport phenomena between both neoteric phases in order to improve the efficiency of these reaction systems. By using two similar ILs based on the same ions, but with different degrees of hydrophobicity in the cation, [Btma][NTf_2] and [CNPrtma] [NTf_2], the continuous synthesis of six different short chain alkyl esters (*e.g.* from butyl acetate to octyl propionate) catalyzed by CALB in $scCO_2$ was studied. Using Hansen's solubility parameter (δ) as criterion to compare the hydrophobicity of the main alkyl chain of cations in ILs with respect to those of substrates and products, it was shown how the same values for this parameter in reagents and IL resulted in a clear improvement of productivity, as a consequence of favouring the mass-transfer phenomena between both the IL and the $scCO_2$ phases.[46b]

A further step towards green biocatalysis in IL–$scCO_2$ biphasic systems was the appropriate selection of acyl donor in the CALB-catalyzed kinetic resolution (KR) of *rac*-1-phenylethanol, because the selective separation of the synthetic product can be included as an integrated step in the full process.[47] By using vinyl laurate as acyl donor, the stereoselective synthetic product, (*R*)-(1-phenylethyl)laurate, can be selectively separated from the non-reacted alcohol with $scCO_2$. This

process takes advantage of the fact that the solubility of a compound in $scCO_2$ depends on both the polarity and vapour pressure. Thus, if the alkyl chain of an ester product is long enough, its low volatility should mean that it is less soluble in $scCO_2$ than the corresponding alcohol. Using this strategy, the introduction of an additional separation chamber placed between the reactor and the backpressure outlet of the system (as example see Fig. 5B), and the selection of an appropriate pressure and temperature resulted in the selective separation of the synthetic product from the reaction mixture (66% yield, >99.9% ee). Other examples of selective extraction of products by $scCO_2$ after lipase-catalyzed transesterification IL media are the butanolysis of triolein in [Toma][TFA],[48a] the KR of 2-octanol in [Omim][PF_6] and [Omim][NTf_2],[48b] and the synthesis of citronellyl laurate in [Bmim][PF_6] and [Bmim][BF_4].[48c] In the case of butanolysis of triolein, the enzymatic reactions first occur in 80% v/v [Toma][TFA] as a homogeneous liquid phase. Then, the butyl oleate product is extracted in a second step using $scCO_2$ at 85 bar and 35 °C, even though the authors did not previously study the phase behaviour of the system.[48a] However, in the case of *rac*-2-octanol, authors first studied the vapor–liquid equilibrium data for systems containing IL, $scCO_2$ and reaction products of a lipase-catalyzed kinetic resolution of *rac*-2-octanol, by using succinic anhydride as acylating agent. By means of these experiments, the partition coefficients of the reaction products between the IL-rich phase and the CO_2-rich phase were calculated. Then, the post-reaction mixture was placed in $scCO_2$ at 11 MPa and 35 °C, which allowed the unreacted enantiomer of 2-octanol to be completely recovered with a very high enantiomeric excess (98.42%).[48b] In another interesting example, the solubility of the enzyme *Candida antarctica* lipase B (CALB) the IL 1-hydroxy-1-propyl-3-methylimidazolium nitrate was studied. This work showed how pressurization with CO_2 in supercritical conditions (35–70 °C, 120 bar) did not produce precipitation of the enzyme. Also, at constant CO_2/IL ratios, the pressure of the bubble points remained almost unchanged at all the assayed enzyme concentrations, while the recovery of the pure IL was made possible by precipitating the enzyme using 2-propanol as an anti-solvent.[48d]

Enzymes other than lipases, *e.g.* cutinase from *Fusarium solani pisi* immobilized onto zeolite NaY, were also tested in a [Bmim][PF_6]–$scCO_2$ system to carry out the KR of 2-phenyl-1-propanol. The results pointed to the protective effect of the IL against enzyme deactivation by $scCO_2$, as well as higher activity than that observed for the cutinase–IL system. This enhanced activity was attributed to the CO_2 dissolved in the IL, which would decrease its viscosity and hence improve the mass transfer of substrates to the enzyme active site.[49]

Further highlighting the excellence of IL–$scCO_2$ biphasic systems, two final approaches are worth mentioning because of improvements introduced in the development of these systems for industrial purposes, such as multicatalytic processes and reaction systems with a reduced amount of ILs. Integrated multicatalytic processes, whereby one initial substrate is catalytically transformed into one final product by two or more consecutive catalytic steps in the same reaction system, is of great interest for developing new chemical industries. In actual fact, a living cell may be considered the most complete multicatalytic system able to produce hundreds of compounds from only one substrate (*e.g.* glucose), which is why the term 'cell factory' is often referred to.[50] On the other hand, some ILs have been described as being not fully green solvents because of their low biodegradability and high (eco)toxicological properties,[51] so that reaction systems based on reduced amounts of ILs are preferred.

Enzyme-catalyzed kinetic resolution (KR) is probably the most widely used method for separating the two enantiomers of a racemic mixture, the chemical yield of the process being limited to 50%. However, this drawback can be overcome by combining the enzymatic KR with *in situ* racemization of the undesired enantiomer, using so-called dynamic kinetic resolution (DKR), which theoretically permits the attainment of 100% of one enantiomeric product (see Fig. 8A). As a first example, the DKR of *rac*-1-phenylethanol was carried out by combining immobilized lipase (Amano PS CI) with a chemical catalyst (either the metal catalyst [Ru(*p*-cymene)Cl_2]$_2$, or the acid catalyst Nafion SAC 13) in a discontinuous way, and without the presence of ILs.[52] The immobilised lipase displayed excellent yields (48–49%) and enantioselectivities (98–99%) to give (*R*)-phenylethyl acetate product in $scCO_2$ at 100 bar and 40 °C. The combination of the immobilized lipase with a chemical catalyst allowed the yield of the (*R*)-product to be improved: 70% with a Ru-catalyst; 85% with a Nafion-catalyst compared to the reaction carried out in hexane (30–35% yield). The enantioselectivities of the products were slightly higher in $scCO_2$ (96% ee with the Ru-catalyst and 85% ee with Nafion) than in hexane (91% ee and 81% ee, respectively). The moderately low enantioselectivities achieved for the acidic Nafion were attributed to the uncontrolled chemical esterification of the substrates catalysed by this solid acid, so that a physical separation of the enzyme and the chemical catalyst, along with the use of a continuous flow system, is suggested as a way to prevent this undesirable side reaction.

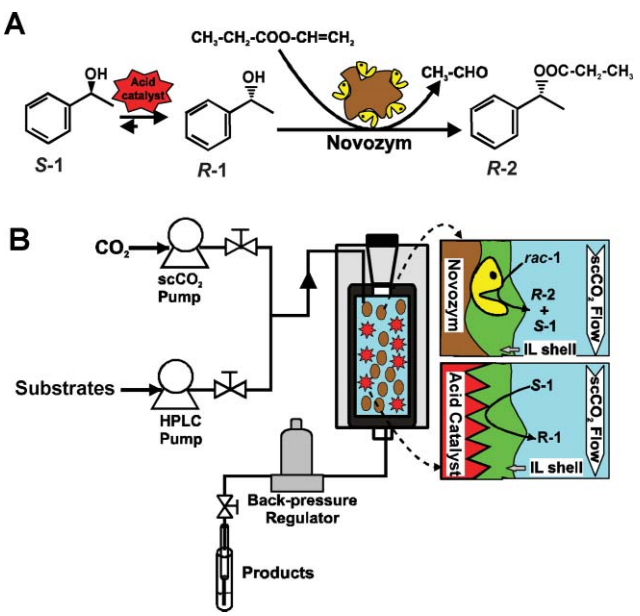

Fig. 8 **A**. DKR of *rac*-1-phenylethanol (*rac*-**1**) catalyzed by the combined action immobilized CALB (Novozym 435) and a solid acid catalyst (*e.g.* zeolite). **B**. Set-up of a continuous packed-bed reactor containing both Novozym 435 and zeolite coated with ILs.[53b]

In this context, continuous DKR processes of *rac*-1-phenylethanol were carried out combining immobilized CALB with silica modified with benzenosulfonic acid groups as catalysts in a packed-bed reactor under $scCO_2$ at 50 °C and 100 bar. Both the chemical and the enzymatic catalysts were previously coated with ILs (*e.g.* [Emim][NTf_2], [Btma][NTf_2] or [Bmim][PF_6]) at a 1 : 1 (w:w) ratio, to prevent enzyme deactivation by $scCO_2$.[53a] However, the use of both catalysts as a simple mixture resulted in a complete loss of activity, probably due to the acid environment around the enzyme particles, which would lead to deactivation. Consequently, it was necessary to pack catalyst particles in three different layers (immobilized enzyme–acid catalyst–immobilized enzyme) physically separated by glass wool to obtain encouraging results for the (*R*)-ester product (76% yield, 91–98% ee).[53a] For this reactor configuration, the (*R*)-ester product yield may only approach 100% if several enzymatic and acid catalyst layers are stacked in the packed bed, according to a dichotomist progression. It is also worth noting how the presence of the undesired (*S*)-ester and hydrolytic products in the $scCO_2$ flow was enhanced when the acid catalyst particles were used without an IL coating.

The use of weak solid acids, such as zeolites, as a chemical catalyst clearly improved the results. Four different acid zeolites coated with ILs ([Bmim][PF_6], [Bdmim][PF_6], [Odmim][NTf_2], [Toma][NTf_2] and [Btma][NTf_2]) were able to perform the racemisation of (*S*)-1-phenylethanol, and their suitability to perform the continuous DKR of *rac*-1-phenylethanol in combination with immobilized CALB under $scCO_2$ flow was successfully demonstrated (see Fig. 8B).[53b] The best results (98% yield, 95% ee) were obtained for a heterogeneous mixture between fajausite-type zeolite (CBV400) particles coated with [Btma][NTf_2] and Novozym particles coated with the same IL. Due to the low acidity of the assayed zeolites, the packaging of the heterogeneous mixture of catalyst particles coated with IL did not result in any activity loss of the immobilized CALB during 14 days of continuous operation in CO_2 under different supercritical conditions (see Fig. 9). This work clearly demonstrates the exciting potential of multi-catalytic (enzymatic or chemo-enzymatic) systems in ILs–$scCO_2$ for synthesizing optically active pharmaceutical drugs by a sustainable approach.

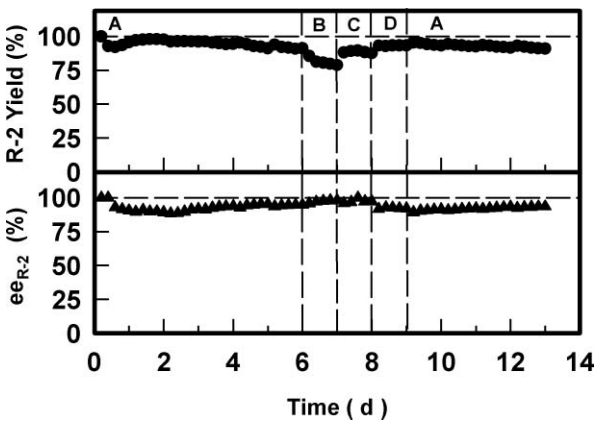

Fig. 9 Operational stability of continuous DKR of *rac*-1-phenylethanol catalyzed by both Novozym 435 and zeolite CBV400 coated with [Btma][NTf_2] in $scCO_2$ at (A) 50 °C and 100 bar, (B) 50 °C and 120 bar, (C) 60 °C and 120 bar and (D) 60 °C and 100 bar. Reproduced by permission of the Royal Society of Chemistry.[53b]

A further step towards reducing the amount of ILs used in catalytic processes in IL–$scCO_2$ biphasic systems arose from the development of solid supports, to which the IL phase is adsorbed or covalently attached.[54] By this approach, IL properties are transferred to the solid phase, leading to either particles or a monolithic supported ionic liquid-like phase (SILP), which have been successfully used in several processes, such as the Henry reaction,[54a] Friedel–Crafts alkylation of cumene,[54b] desulfurization of fuels[54c] and solid-phase extraction.[54d] In the case of enzyme catalysis, there have been reports of the immobilization of *Candida rugosa* lipase on magnetic nanoparticles with supported ILs, based on imidazolium cations with different chain lengths (C_1, C_4 and C_8) and anions ([Cl], [BF_4] and [PF_6]). These new immobilization supports were obtained by covalent bonding of ionic liquid–silane moieties on magnetic silica nanoparticles (55 nm diameter), which permits large amounts of lipase to be loaded (about 64 mg per 100 mg carrier). Furthermore, the activity of bound lipase was 118.3% compared to that of the native lipase, when the esterification of oleic acid with butanol in free solvent media was used as activity test at 30 °C.[55a] In another example of enzyme catalysis in $scCO_2$ (see Fig. 10), bioreactors with covalently supported ionic liquid phases (SILP) were prepared as polymeric monoliths based on styrene–divinylbenzene, containing imidazolium units in loadings ranging from 54.7 to 39.8 wt% IL per gram of polymer, which results in a liquid phase coating the surface of the solid support. The SILPs were able to absorb 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 by transesterification in $scCO_2$ at 100 bar and 40–100 °C. The catalytic activity of these mini-flow bioreactors remained practically unchanged for seven operational cycles of 5 h each in different supercritical conditions.[55b]

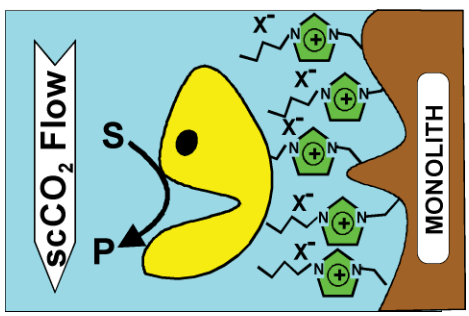

Fig. 10 Scheme of immobilized enzyme onto monolith-supported IL phase (SILP) in continuous operation under supercritical conditions.[55b]

In the same context, the discovery that poly(ethylene)glycol (PEG) and $scCO_2$ formed similar biphasic systems to that of IL–$scCO_2$, where PEG has very low solubility in $scCO_2$ but CO_2 is highly soluble in PEG, has opened a new alternative to ILs for the development of this kind of enzymatic biphasic process.[56] Furthermore, PEG is less expensive than ILs and is considered an acceptable additive for food with a fully evaluated toxicity

value. Using the lipase-catalyzed kinetic resolution of *rac*-1-phenylethanol by vinyl acetate as a reaction model, preliminary studies showed the suitability of this PEG–$scCO_2$ biphasic medium, which maintained enzyme activity and selectivity at the highest level (50% yield and 98% ee) after 11 cycles at 50 °C and 80 bar.

5 Multiphase biocatalytic systems based on FSs

Biocatalysis in FSs is a recent area of research, although few papers have been published. The unique property of FSs (*e.g.* perfluorohexane) to be miscible or immiscible with organic solvents (*e.g.* hexane) as a function of the temperature is the key concept for developing multiphase synthetic processes. The operational strategy begins when a catalyst is dissolved in a fluorous solvent and combined with substrates dissolved in the organic solvent to form a biphasic system. By warming the reaction system, the two phases become miscible and form one phase. The catalytic reaction can then occur in a homogeneous system and, finally, products can easily be recovered by simple re-cooling of the reaction mixture, because the two phases separate and the catalyst remains in the fluorous phase ready to be reused in another cycle.[8b,9]

In a pioneering example, the enantioselective esterification of *rac*-2-methylpentanoic acid with highly fluorinated decanol catalyzed by *Candida rugosa* lipase in a perflurohexane–hexane biphasic system has been reported (see Fig. 11).[57] The acid substrate is dissolved in hexane, while the fluorinated alcohol is dissolved in the fluorous phase. When the resulting biphasic mixture is warmed, becoming one phase, the lyophilized enzyme is added to perform the reaction. The lipase selectively catalyzes the esterification of (*S*)-2-methylpentanoic acid to the corresponding (*S*)-fluorinated ester product. At the end of the reaction, the biocatalyst is separated by filtration, and the re-cooling of the reaction mixture results in the retention of the fluorinated product into the fluorous phase, while the unreacted (*R*)-2-methylpentanoic acid remains in the hexane phase. An important problem of this strategy is the need to use substrate(s) miscible in the fluorous phase, as well as the long reaction time required (95–145 h) to reach 49–53% conversion and 95% ee for the (*S*)-product. Furthermore, as enzymes are not soluble in FSs, a key step of this approach, such as the reuse of the catalyst dissolved in the fluorous phase for continuous operation cycles, cannot be carried out. In this context, Poliakoff and Thomas's group reported an elegant approach based on the rule "like dissolves like", which permits the solubilisation of enzymes in FSs and $scCO_2$.[58] By using perfluoro anionic surfactants, *e.g.* perfluoropolyether carboxylate (Krytox, see Fig. 11B), the extraction of cytrochrome C (Cc), α-chymotrypsin (CT) and *Candida rugosa* lipase (CRL) from aqueous solutions into a FS, *e.g.* perfluoromethylcyclohexane (PFMC) has been demonstrated: the anionic surfactant is able to interact by hydrophobic ion pairing (HIP) with basic amino acid residues (*e.g.* Lys, His, Arg) placed on the surface of a protein dissolved in aqueous buffer at pH below its isoelectric point. Thus, the HIP–protein complexes containing the perfluoro anionic surfactant can easily be extracted into perfluoromethylcyclohexane, resulting in a homogeneous and clear phase which contains up to 20 mg Cc per mL or 0.8 mg CT per mL, respectively. Spectroscopic studies of ion paired Cc–Krytox and CT–Krytox indicate that both proteins retain their α-helical secondary structures, while the CT–Krytox complex retain its catalytic activity over four reaction cycles in a fluorous biphasic systems. Additionally, the CT–Krytox complex was also found to dissolve and be catalytically active in $scCO_2$ at 172 bar and 40 °C.[58a] By using the transesterification of *N*-acetyl-L-Phe ethyl ester with n-butanol or *rac*-2-butanol as catalytic test, the activity of the CT–Krytox complex in PFMC (6–10%) was shown to be significantly higher than that of the suspended protease (1–3%) in either the FBS hexane–PFMC or $scCO_2$. Furthermore, the CT–Krytox complex, which is retained in the fluorous phase on cooling the solution, was successfully reused over four cycles with no loss of activity.[58b] In the CRL case, the CRL–Krytox complex formed a highly stable colloidal dispersion in both liquid and supercritical carbon dioxide at high CO_2 densities (>0.92 and 0.847 g mL^{-1}, respectively), with 4% by volume PFMC as a cosolvent, yielding an almost transparent orange fluid. Furthermore, a reversible aggregation phenomenon of the enzyme–surfactant complexes in CO_2 was demonstrated at 25 and 40 °C and at various pressures (138–345 bar), with hydrodynamic diameters ranging from 50 to 200 nm. Data concerning the activity and/or operational stability of this complex were not provided.[58c] In another example, the *Bacillus cepacia* lipase–Krytox complex was successfully assayed for the KR of 1-phenylethanol in a PFMC-hexane biphasic system, showing high stereospecificity (*ca.* 99%) with moderate catalytic efficiency (49% yield after 99 h) with a high operational and storage stability. Temperature modulation of the FBS miscibility allowed the separation and

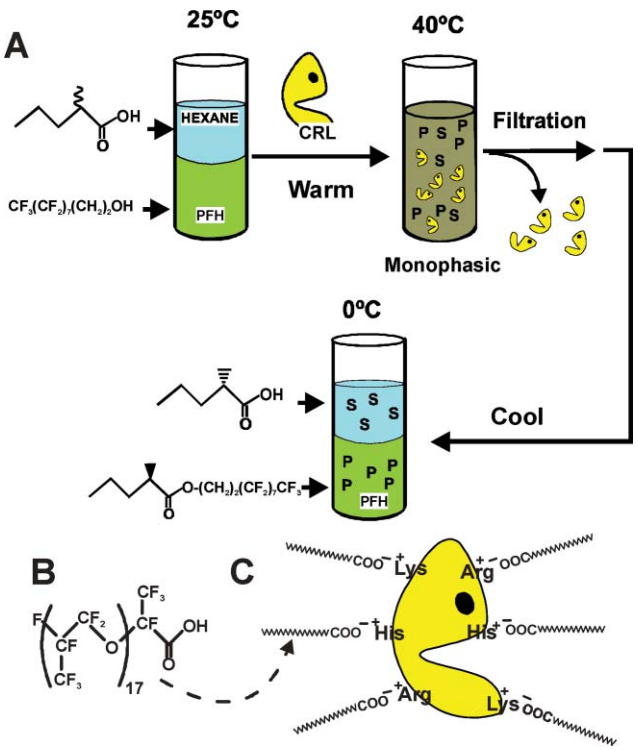

Fig. 11 **A.** General scheme for enantioselective partitioning in lipase-mediated esterification using fluorous biphase methodology. CRL, *Candida rugosa* lipase; PFH, Perfluorohexane.[57] **B.** Structure of perfluoropolyether carboxylate surfactant (Krytox 157 FSL®). **C.** Scheme of an enzyme–Krytox complex formed by hydrophobic ion pairing.[58a,b]

recovery of the solubilised lipase for at least three operation cycles.[58d]

6 Challenges and future outlook

Nature has always been a source of inspiration for chemists. To transfer the exquisite efficiency shown by enzymes in Nature to chemical processes may constitute the most powerful toolbox for developing a clean and sustainable chemical industry in the near future. By using enzymes in non-aqueous environments, rather than their natural aqueous reaction media, their technological applications can be greatly enhanced because of the resulting expansion in the repertoire of enzyme-catalyzed transformations. Consequently, a number of potentially interesting applications of enzymes that are either impossible to use or of marginal benefit in water become commercially attractive in non-aqueous environments.

The unique properties of ILs, which can be tailored by an appropriate selection of ions, has opened the door to a new chemistry because of the processing options that have been made available by using conventional organic solvents. The implementation of ILs in the chemical industry offers the opportunity to drastically modify all chemical processes based on these conventional solvents in the near future. Additionally, the exceptional ability of some ILs to overstabilize enzymes, which maintain their native and active conformation even under extremely harsh conditions, is an important added value of ILs in the construction of a sustainable chemical industry.

Although FSs are still not fully accepted as green/clean solvents, the complexation of enzymes with fluorinated ionic surfactants seems to improve the properties of enzyme–IL systems, because they provide homogeneous reaction systems with active and stabilized enzymes. However, the need to use VOCs to extract reaction products is a clear breakdown in the greenness of this fluorous biphasic approach.

Supercritical CO_2 seems to be the perfect companion of ILs for the development of downstream steps in green synthetic processes, as well as for cleaning and recovering ILs for re-use, because of the unique phase behaviour of IL–$scCO_2$ systems. In the near future, the synthesis and application of new ILs, which may be suitable for maintaining all the excellent characteristics reported above, but which are fully biodegradable and with low or zero toxicity, is one of the most important targets for the development of any sustainable industrial bioprocess.[59] The combination of enzymes with FS–$scCO_2$ or LP–$scCO_2$ could be another attractive strategy to integrate both biocatalytic and extractive steps, although further experiments should be carried out in this respect.

By combining enzymes with multiphase systems, the chemical industry has a clear strategy for developing integral green synthetic processes, where the use of more complex enzymes, (*e.g.* oxidoreductases, lyases, *etc.*) should also be explored. The key to successful implementation of all these catalytic methodologies in chemical manufacturing is that they combine integration of the catalytic steps in multi-step organic syntheses and downstream processing without the need to isolate intermediates, mimicking the metabolic pathways found in Nature. Multi-enzymatic and/or multi-chemoenzymatic green chemical processes in multiphase systems for synthesizing pharmaceutical drugs are only a beginning, but the door for the development of a sustainable chemical industry is open.

Beyond this context, it might also be mentioned how the exceptional behaviour of enzymes in IL–$scCO_2$ systems could also be used to hypothesize about liquid media (other than water), capable of maintaining biomolecules active in alternative forms of life on other planets that clearly show abiotic conditions.

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