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# Activation and stabilization of enzymes in ionic liquids

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As environmentally benign "green" solvents, room temperature ionic liquids (ILs) have been used as solvents or (co)solvents in biocatalytic reactions and processes for a decade. The technological utility of enzymes can be enhanced greatly by their use in ionic liquids (ILs) rather than in conventional organic solvents or in their natural aqueous reaction media. In fact, the combination of green properties and unique tailor-made physicochemical properties make ILs excellent non-aqueous solvents for enzymatic catalysis with numerous advantages over other solvents, including high conversion rates, high selectivity, better enzyme stability, as well as better recoverability and recyclability. However, in many cases, particularly in hydrophilic ILs, enzymes show relative instability and/or lower activity compared with conventional solvents. To improve the enzyme activity as well as stability in ILs, various attempts have been made by modifying the form of the enzymes. Examples are enzyme immobilization onto support materials *via* adsorption or multipoint attachment, lyophilization in the presence of stabilizing agents, chemical modification with stabilizing agents, formation of cross-linked enzyme aggregates, pretreatment with polar organic solvents or enzymes combined with suitable surfactants to form microemulsions. The use of these enzyme preparations in ILs can dramatically increase the solvent tolerance, enhance activity as well as stability, and improve enantioselectivity. This perspective highlights a number of pronounced strategies being used successfully for activation and stabilization of enzymes in non-aqueous ILs media. This review is not intended to be comprehensive, but rather to present a general overview of the potential approaches to activate enzymes for diverse enzymatic processes and biotransformations in ILs.

## 1. Introduction

Room temperature ionic liquids (ILs) have emerged as a potentially attractive "green"[1]recyclable alternative to environmentally harmful organic solvents.[2] Ionic liquids are composed entirely of ions (generally consisting of organic cations, namely derivatives of *N*,*N*′-substituted imidazolium, *N*-substituted pyridinium, tetraalkylated ammonium, and tetraalkylated phosphonium, and either organic or inorganic anions), and are liquids at ambient or far below ambient temperatures, a property required for many different chemical reactions. In essence, ILs have negligible vapour pressure and are nonflammable,[3] and so are not readily lost to the

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environment. Not only are ILs more environmentally attractive than volatile organic compounds, but they possess many unique and attractive physicochemical properties, including multiple solvation interactions with organic and inorganic compounds,[4a] excellent chemical and thermal stability,[4b] high ionic conductivity[4c] and a large electrochemical window. Importantly, the enormous diversity of ILs makes them suitable for a number of industrial applications.[5] In fact, the physicochemical properties of ILs, such as viscosity, hydrophobicity, density and solubility can be tuned by simply selecting different combinations of cations and anions, as well as attached substituents, leading to the use of the terms "designer" and "task-specific" ILs.[6]

For more than a decade, ILs have been increasingly exploited as solvents and/or (co)solvents and/or reagents in a wide range of applications due to their superb properties: the combination of green properties with their tailor-made chemical, physical and biological properties. In this respect, ILs have been shown to have a significant advantage over conventional solvents for use in chemical engineering (*e.g.*, separation, extraction and membranes), chemistry (*e.g.*, organic synthesis, catalytic reactions, nanomaterial synthesis and polymerization reactions), energy conversion (*e.g.*, batteries, fuel cells and heat storage) and biotechnology (*e.g.*, biocatalysis, biomolecules purification and biofuel production), and their applications continue to expand. In particular, environmentally benign ILs[1] as "green" non-aqueous reaction media for enzyme-catalyzed reactions provide numerous synthetic and processing advantages (Box 1).[7]

**Box 1. Advantages of using enzymes in ILs media**

- Scope to design particular bioprocesses due to the designable physical/chemical properties of ILs.
- In most cases, enhanced operational and thermal stability.
- Increased solubility of sparingly soluble substrates.
- Alteration of substrate specificity.
- Ease of enzyme recovery simply by filtration or centrifugation.
- Ease of substrate and product recovery simply by evaporation and/or mixing with non-polar organic solvents and/or supercritical $CO_2$.
- Suppression of water dependent side reactions.
- Ease of immobilization of enzyme in highly viscous ILs.

## 2. Biocatalysis in ionic liquids

The first example of using an enzyme in an IL was reported in 1984 when Magnuson and co-workers demonstrated the activity and stability of alkaline phosphatase in aqueous mixtures of $[EtNH_3][NO_3]$.[8] Unfortunately, this finding did not attract significant attention due to the lack of knowledge of ILs at that time. Encouragingly, in 2000, several reports on enzyme-catalyzed reactions in ILs appeared within a few months.[9] Among these, the first whole cell biocatalysis in ILs was conducted using *Rhodococcus* R312 in a biphasic $[bmim][PF_6]$-water system[9b] (bmim = 1-butyl-3-methylimidazolium). Russell and co-workers[9a] reported the first biotransformation in IL $[bmim][PF_6]$ containing 5% (v) water using the protease thermolysin as a biocatalyst for synthesis of *Z*-aspartame. Sheldon *et al.*[9c] have shown several synthetic reactions catalyzed by free and immobilized CaL-B in two anhydrous ILs $[bmim][PF_6]$ and $[bmim][BF_4]$. Since then, a wide number of enzymes have been examined in ILs to test their catalytic activity; examples are lipases,[9c,10] proteases,[9a,11] oxidoreductases,[12] peroxidases,[13] whole cells,[9b,14] and so forth. The use of enzymes in ILs has presented many advantages, such as high conversion rates, high enantioselectivity, and better enzyme stability,[9a,10j,10k,15] as well as better recoverability and recyclability. In addition to these advantages, ILs can be used for carrying out biotransformations with polar or hydrophilic substrates such as amino acids and carbohydrates which are insoluble or sparing soluble in most organic solvents (*e.g.*, isooctane and hexane).[16]

A number of reviews that focus on enzyme-catalyzed reactions in ILs have appeared since 2002 documenting the advances of the entire field[7] or of specific research groups.[17] An excellent review written by Yang and Pan describes the solvent properties of ILs (*e.g.*, water content, pH, hydrophobicity and impurities), and their effects on enzyme performance, such as activity, stability and selectivity.[7a] Recently, Rantwijk and Sheldon published a well balanced and comprehensive review that extensively covers the various issues associated with biocatalysis in ILs.[7b] Bogel-Łukasik highlighted important results on the enzymatic separation of secondary alcohols *via* lipase-catalyzed esterification in ILs.[17f] This review will not focus on biocatalysis in ILs using commonly used crude enzymes, as there are already excellent reviews available.[7,17] Instead we will focus on the activation and stabilization of enzymes in ILs by their immobilization and/or modification, which is gradually improving the realization of the advantages of ILs for non-aqueous enzymology.

## 3. Activation and stabilization of enzymes in ionic liquids

As discussed in the above sections, the utilization of enzymes in ILs has many potential advantages over conventional organic solvents

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or their natural aqueous reaction media. However, in many cases, the application of enzymes in ILs has been limited by the low solubility, activity and stability of the commonly used lyophilized enzymes (generally called 'free enzyme' or 'crude enzyme'). This is not surprising, because lyophilized enzymes have conformations which usually differ from their native structures, and these enzymes have evolved naturally to function in aqueous environments. In fact, these conformational changes are reversed when lyophilized enzymes are dissolved in water, while they remain unchanged when enzymes are used as powders in ILs. In addition, most of the hydrophilic ILs can remove (or strip) enzyme-bound water molecules that are crucial for maintaining enzyme structure and function. Nevertheless, it is well established that enzymes can function in ILs with and without water. However, improving enzyme function in these new non-aqueous solvents is required for large-scale, economically viable biotransformation.

To improve enzyme activity in IL environments, strategies ranging from biocatalyst formulations to tailoring of the reaction medium to a particular reaction have been applied with varying degrees of success. In an attempt to overcome the insolubility of enzymes, a number of groups have synthesized task-specific ILs by introducing functional groups such as hydroxyl, ether, and amide groups that show high affinity for protein molecules. Some of these hydrophilic ILs are found to solubilize enzymes *via* weak hydrogen bonding interactions;[10i,m,13d,15h,17e] however, most of them inherently cause conformational changes in enzymes, resulting in inactivation.[10i,17e] The large loss in enzyme activity seen in hydrophilic ILs (such as those based on chloride, acetate, nitrate and dicyanamide anions) is attributed to the interactions between anions and enzyme since this loss is not observed when enzymes were suspended in hydrophobic ILs. Another approach to improve enzyme solubility in ILs is to add a small amount of water; however, the dissolved enzymes typically show low catalytic activity due to their changed conformation in ILs.[16b] Recently, several researchers have reported enzymatic reactions in hydrophilic ILs with a high concentration of added water,[13a,d,18] although such homogeneous media are far from the ideal of non-aqueous enzymology. In addition, most work on enzymatic catalysis in IL media so far has been conducted using lipases as biocatalysts because most other crude enzyme preparations were found to be inactive in ILs. To provide more applicable systems of enzymes in ILs, various enzyme formulation and solvent engineering approaches have been developed and tested.

Based on the existing knowledge of enzyme behavior in non-aqueous media, a number of strategies have been described in the literature to obtain different enzymes preparations that can catalyze reactions in ILs with significant efficiency. Examples include the use of physically or chemically immobilized enzymes, cross-linked enzyme aggregates (CLEAs), organic solvent treated enzymes and so forth. Most of these treatments not only preserve the facile recovery advantages of enzymes but also avoid deleterious enzyme aggregation by spreading the enzymes on the support surface. In addition to the enzyme formulation, tailoring the reaction medium is another potential way to activate enzymes in ILs. In this approach, the formation of microemulsions in ILs has emerged as a potential template for improving the enzyme microenvironment in a non-aqueous IL environment. This review brings together details of such recent approaches to activate enzymes for use in IL media, focusing particularly on the use of enzyme preparations that result in dramatic improvements in biocatalysis.

### 3.1 Enzyme formulations

**3.1.1 Immobilized enzymes on support materials.** Immobilization of enzymes offers many advantages for biocatalysis in harsh conditions: convenient separation for reuse after the reaction by filtration, centrifugation, *etc.*; application in continuous processes; easy product separation and recovery; and stabilization of enzymes. The advantages of using immobilized enzymes in non-aqueous media such as organic solvents[19] and supercritical fluids[20] are well-documented. Hence, one would expect that immobilization of biocatalysts should improve biotransformations conducted in ILs compared with free enzymes. To date, a wide range of commercially available enzymes immobilized on solid supports, such as Novozym 435 (CaL-B), Lipozyme (RML), Chirazymes [*Candida antarctica* lipase A (CaL-A), CaL-B, and *Psedumonas cepacia* lipase (PCL)], Lipolases [*Aspergillus oryzae* lipase (AOL), and *Humicola lanuginose* lipase (HLL)], have been examined for catalytic activity in ILs.

*Candida antarctica* lipase B (CaL-B, *i.e.*, Novozym 435) immobilized on an acrylic resin has an excellent ability to catalyze a wide range of reactions in ILs, such as transesterification,[9c,10d,10k,m,21,22] ammonolysis,[9c] acylation of sugars,[10d,23] and polymer synthesis.[24] Although the free lipase showed transesterification activity even in anhydrous ILs, the reaction catalyzed by Novozym (NZ) 435 was much faster than that of the free lipase.[10k] Significantly, CaL-B shows more thermal and operational stability in ILs than when suspended in common organic solvents.[9c,10k,10m] For an example, the operational half-life of CaL-B in a series of ILs $[C_nMe_3N][Tf_2N]$ (where, $C_nMe_3N$ = alkyltrimethylammonium and $Tf_2N = (CF_3SO_2)_2N^-$) was up to 2000 times greater than that in hexane.[10m] Fluorescence and CD spectroscopy studies indicated that the conformational stability of CaL-B was much greater in $[emim][Tf_2N]$ and $[buMe_3N][Tf_2N]$ at 50 °C than in water or hexane.[15e,g] Furthermore, lipase PS (commercial trade name of *Psedumonas cepacia* lipase) is also commercially available from Amano Enzyme Inc as an immobilized form on Celite, and has been shown to give better activity in ILs.[10e,10l,25] In essence, swelling of the carrier in ILs renders the enzyme more accessible to the solvent.[10j]

In addition to the commercial immobilized lipases, many research groups have immobilized enzymes on some solid supports, yielding various degrees of enzymatic catalysis in ILs. Among various methods reported, enzyme adsorption on supports is a relatively simple and inexpensive method of immobilization and is regularly used in large scale processes, particularly where the enzyme is inexpensive.[26] It was found that no activity could be detected in ILs when native esterases were used as biocatalysts.[11c,27] However, two esterases from *Bacillus subtilis* and *Bacillus stearothermophilus* immobilized by adsorption onto Celite were shown to catalyze the transesterification of 1-phenylethanol in ILs with similar activity and enantioselectivity to that seen in organic solvents (see Table 1).[27] Importantly, in IL reaction media the half-life of immobilized enzymes was increased by 30 times as compared to hexane. Similar promising results were reported when $\alpha$-chymotrypsin immobilized on Celite was employed for

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**Table 1** Specific activity, enantioselectivity and stability of the esterase from *Bacillus stearothermophilus* immobilized onto Celite in ILs and organic solvents[27]

| Solvent[a] | Specific activity (U/g protein) | *E*-value | Half-life/h |
|---|---|---|---|
| [bmim][$PF_6$] | 0.14 | 3.5 | >240 |
| [bmim][$BF_4$] | 0.33 | 3.5 | 220 |
| [bmim][BTA] | 0.36 | 2.9 | — |
| MTBE | 0.29 | 2.8 | 80 |
| Hexane | 0.59 | 3.9 | 8 |

[a] No activity was observed for free enzyme in ILs.

catalyzing transesterification of *N*-acetyl-L-phenylalanine ethyl ester (APEE) with 1-butanol in [emim][$Tf_2N$] and [bmim][$Tf_2N$].[16b]

In another approach, Itoh *et al.* immobilized lipase PS on inorganic materials such as metal oxide, ceramics and mesoporous silica, and tested their activity for the kinetic resolution of methyl (±)-mandelate in the commonly used ILs [bmim][$PF_6$], [bmim][$BF_4$] and [bmim][$Tf_2N$].[28a] Although the enhancement in the reaction rate was shown to depend significantly on the combination of the source of the supporting materials and solvent system, lipase immobilized with tungsten(VI) oxide coated-metal oxide ($WO_3/M_xO_y$) was found to be suitable in ILs having different anions. The activity and selectivity of immobilized lipase in ILs are comparable to that found in *i*-propanol. The stabilization of lipase was also achieved by immobilization on the ceramic Toyonite carriers, which are now commercially available from Amano Enzyme Ltd.[28b,c] Barreiros *et al.* reported that cutinase immobilized on zeolite NaY for the transesterification of (*R*,*S*)-2-phenyl-1-propanol by vinyl butyrate in ILs shows good activity.[20c]

Recently, enzyme–carbon nanotube conjugates were used in ILs and found to afford very high retention of biological activity.[29] Dordick *et al.* reported that the simple adsorption of proteinase K onto single-walled carbon nanotubes (SWNTs) provides highly active, thermally stable and reusable biocatalyst formulations compared to the native suspended enzyme counterpart.[29a] More importantly, proteinase K-SWNT conjugates are found to be far less diffusionally limited than the native enzyme suspended in ILs. This effect could be very useful to overcome key operational limitations in viscous ILs. Similar promising results were observed when multiwalled carbon nanotubes (MWNTs) were used as the supports for immobilization of lipases from *Candida rugosa* (CRL).[29b] Lipase-MWNT conjugates catalyzed the transesterification of (±)-1-phenylethanol with vinyl acetate in ILs with markedly higher conversions than those observed for the free enzymes (see Table 2). It was suggested that the interaction with the hydrophobic surface of the nanotubes causes a conformational changes in the lipase molecule to the "open lid" structure.

The immobilization of CRL on magnetic DEAE-GMA-EDMA (diethylamino ethylglycidyl methacrylate-ethylene glycol dimethacrylate) microspheres by ion exchange and examination of the enzymatic activity of the conjugate in [bmim][$PF_6$] has been investigated recently.[30] Interestingly, this immobilized lipase displayed a "pH memory" in ILs. This phenomenon is very common for enzymes in organic solvents: enzyme catalytic activity reflects the pH in the last aqueous solution to which they were exposed.[31] Furthermore, the pH was shown to have a significant influence on the conversion and the enantioselectivity of immobilized CRL-catalyzed resolution of (±)-menthol in [bmim][$PF_6$]. Good

**Table 2** Transesterification of (±) 1-phenylethanol in IL[bmim][$PF_6$] with *Candida rugosa* lipase immobilized on MWNTs[29b]

(Scheme: 1-phenylethanol + vinyl acetate —Lipase/ ILs→ (OH) + (OAc))

| Lipase formulations | Time/h | Conversion (%) | ee (%) | *E*-value |
|---|---|---|---|---|
| CRL adsorbed on MWNTs | 12 | 17 | >99 | 250 |
| | 24 | 34 | >99 | 350 |
| | 36 | 37 | >99 | 360 |
| Free CRL | 12 | 1 | — | — |
| | 24 | 2 | — | — |
| | 36 | 5 | — | — |

conversion and the best enantioselectivity were obtained with the immobilized lipase prepared at pH 5.0, while the enantioselectivity decreased gradually with increasing pH value.

In an attempt to solubilize proteins in ILs, our group has described the formation of protein–crown ether complexes that dissolve in ILs and catalyze homogenous reactions.[32] In this process, the Lys-rich protein cyt-*c* was extracted into the hydroxyl-containing hydrophobic ILs (*e.g.*, [$C_2$OHmim][$Tf_2N$]) with crown ethers *via* supramolecular complexation (see Fig. 1). Crown ether dicyclohexano-18-crown-6 (DCH18C6), which is highly soluble in ILs, was used as the extractant. The interactions between DCH18C6 and the amino groups ($NH_3^+$) in the lysine residues on the protein surface was described as the main driving force for the cyt-*c* extraction into ILs. The extracted cyt-*c*-DCH18C6 complex exhibited improved peroxidase activity for the oxidation of 2,6-dimethoxyphenol (DMP) in [$C_2$OHmim][$Tf_2N$]: the initial reaction rate was increased by 5.5-fold relative to that of native cyt-*c*.[32a] This result was explained by an enhancement of the enzymes' affinity for $H_2O_2$. Unexpectedly, the half-life of the cyt-*c* complex was decreased by 3 times.

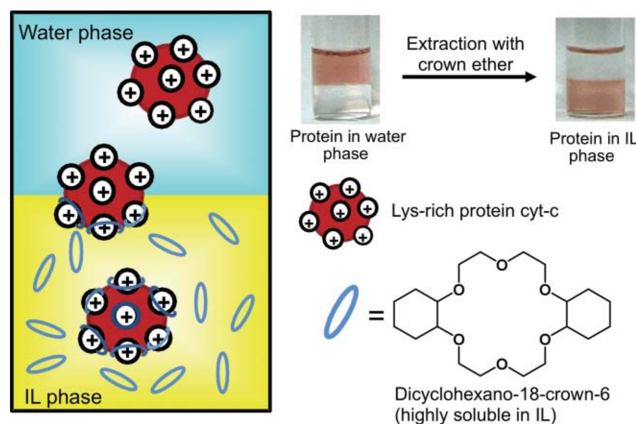


**Fig. 1** Schematic illustration of Lys-rich protein extraction with DCH18C6 from aqueous phase into IL phase, taken from our previous study.[32a]

### 3.1.2 Enzyme lyophilization with stabilizing agents.

One of the most useful methods for activating and stabilizing enzymes in organic solvents is co-lyophilization with structure-preserving lyoprotectants/cryoprotectants such as inorganic salts, sorbitol and poly(ethylene glycol) (PEG).[33,34] Since enzymes are not soluble

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**Table 3** Selected examples of the improvement of enzymatic rates in ILs when enzymes are lyophilized with inert materials

| Entry | Enzyme | Supporting material | Reaction | Initial rate (mM $h^{-1}$ $g^{-1}$ protein) | Ref. |
|---|---|---|---|---|---|
| 1[a] | Lipase PS | PEG-20000 | 2-phenyl-1-propanol + vinyl acetate →2-phenyl-1-propyl acetate | Native = 0.8<br>Complex = 24 | 10*g* |
| 2[a] | Lipase PS | PEG-20000 | vinyl cinnamate + benzyl alcohol → benzyl cinnamate + cinnamic acid | Native = 0.3<br>Complex = 1.07 | 35 |
| 3[b] | Substilisin | a. PEG-6000<br>b. Trehalose<br>c. PEG + trehalose (1 : 2 (w:w)) | *N*-acetyl-L-phenylalanine ethyl ester + n-propanol → *N*-acetyl-L-phenyl-alanine propyl ester + ethanol | Native = 10<br>a. Complex = 100<br>b. Complex = 30<br>c. Complex = 320 | 37 |
| 4[b] | Lipase PS | Hydroxypropyl-β-cyclodextrin | (±)-Allethrolone + vinyl acetate → (±)-allethrolone acetate | Native = 1.02<br>Complex = 2.6 | 36b |

[a] IL [omim][$PF_6$] was used as reaction medium. [b] IL [bmim][$PF_6$] was used as reaction medium.

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in most organic solvents, the association of enzymes with such inert supports can greatly affect enzyme activity and stability by influencing the enzyme hydration level as well as dispersion ability. As both ILs and organic solvents are non-aqueous reaction media, many research groups have prepared physical complexes of enzymes with a wide range of stabilizing agents and examined their activity in ILs.[10g,35–37]

Our group investigated the activity of PEG-coated lipases in ILs.[10g,35] The PEG-20000-lipase complex was prepared *via* water-in-oil (w/o) emulsion followed by lyophilization to obtain PEG-coated enzymes. While native lipase PS showed negligible activity in ILs, coated lipase was shown to be considerably more active as shown in Table 3. However, the same treatment of lipases from other sources showed very little effect for the alcoholysis of vinyl cinnamate with benzyl alcohol.[35] In an optimization study, [omim][$PF_6$] was found to be the best reaction medium for the PEG–lipase complex among the various ILs tested.[10g] The authors suggested that the increase in reaction rate may be due to the better dispersion of PEG-lipase complexes in ILs than free lipase suspensions: immobilized lipase provides the lubricity to the enzyme in these solvent systems.[10g] Interestingly, this PEG–lipase complex showed pH memory in ILs, indicating that the lipase activity could be optimized by the treatment with optimum buffer solutions for each lipase. In a subsequent study, Shah and Gupta demonstrated that lyophilization of subtilisin with PEG-6000 increased the rate of transesterification of APEE in [bmim][$PF_6$] by 10 times (compared with pH-tuned lyophilized enzyme activity in the same IL).[37] The same article also reported very promising results with subtilisin lyophilized with trehalose and a mixture of PEG and trehalose (see Table 3, entry 3). The combination of PEG and trehalose incorporated during lyophilization gave 24-fold increase in initial rates as compared to the rates observed for the enzyme lyophilized alone, though it is not clear how these combinations affect the enzyme activity in IL systems.

Cyclodextrins have also been used for enzyme activation in IL media. In 2007, Wang and Mei observed that lipase PS lyophilized with cyclodextrins could be used for the efficient resolution of racemic allethrolone in ILs with markedly higher enantioselectivity than those observed for the native lipase.[36b] The catalytic behavior of the lipase formulations was influenced by the structure of the cyclodextrins and the types of ILs used: lipase-hydroxypropyl-β-cyclodextrin preparation showed the highest conversion rate in [bmim][$PF_6$], whereas lipase-α-cyclodextrin gave the best conversion rate in [bmim][$BF_4$]. Interestingly, catalytic activity increased as the amount of dextrins used during lyophilization increased. This finding was explained by two terms: firstly, cyclodextrins interact with the enzyme to change its conformation, and secondly, the excess cyclodextrins that do not bind to the enzyme may form host–guest complexes, which could relieve the enzyme from substrate and product inhibition.[38] However, the stability of the lipase-cyclodextrin complex was lower than that of native lipase.

Laszlo and Compton[36a] have reported that α-chymotrypsin lyophilized with $K_2HPO_4$, KCl, or PEG can catalyze the transesterification of APEE with *n*-propanol in [bmim][$PF_6$] or [omim][$PF_6$] containing 0.25% water (v/v) and higher. In contrast, no additional water was required when hexane or isooctane was used as the reaction medium. Unexpectedly, these enzyme preparations did not substantially stimulate activity in ILs, unlike that observed in non-polar organic solvents.[34a,b] Importantly, additional water was not needed for the same enzyme formulation activity if supercritical (sc) $CO_2$ was used as a cosolvent with ILs. This result indicates that ILs provide a relatively polar environment, which can be minimized with non-polar sc$CO_2$ to optimize enzyme activity. Another benefit of using sc$CO_2$ was that it can be used to extract the product from ILs,[36a,39] which is discussed in more detail in section 3.2.3.

**3.1.3 Chemical modification of enzymes with activated stabilizing agents.** Poly(ethylene glycol) (PEG) is well-known for its enzyme stabilizing properties. Hence, PEG-modified enzymes have been used extensively for stabilizing enzymes in denaturing environments, particularly in organic media.[40] Since PEG is found to be highly soluble in ILs, PEG-modified enzymes dissolve easily in this medium and catalyze homogenous reactions. In this approach, the research group led by Ohno was the first to demonstrate that heme proteins (*e.g.*, cytochrome-*c* (cyt-*c*) and cytochrome P450 from *Sulfolobus tokodaii* strain 7 (P450st)) modified with activated poly(ethylene oxide) (PEO) chains could be dissolved in [emim][$Tf_2N$] without denaturation.[41] Structural stability was observed by UV-vis and resonance Raman spectroscopy, and the Raman spectrum of PEO-P450st in IL, even at 120 °C was shown to be very similar to that of wild type P450st in water.[41b] Note that for these PEO–protein complexes, at least 10 PEO chains with average molecular weights of over 2000 were required to solubilize the proteins into ILs and keep the redox activity.[41a] Furthermore, a suitable supporting electrolyte is required for the redox reaction of heme proteins to provide an adequate ion size for the active center.

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**Table 4** Activity and stability of $PM_{13}$-enzyme in IL [emim][$Tf_2N$]

| Enzyme | Preparation | Reaction | Reaction conditions | Initial rates (mM $h^{-1}$ $g^{-1}$ enzy.) | Stability[a] | Ref. |
|---|---|---|---|---|---|---|
| Subtilisin | a. Native<br>b. Sub-$PM_{13}$ | *N*-acetyl-L-phenylalanine ethyl- ester + 1-butanol | 40 °C, pH = 9 | a. n.d<br>b. 45–50 | —<br>b. $t_{1/2}$ = 194 | 44*a* |
| CRL | a. Native<br>b. CRL-$PM_{13}$ | 2-phenyl-1- propanol + vinyl acetate | 40 °C, 1.5% (v/v) water | a. n.d<br>b. 28 | CRL-$PM_{13}$ retains its original activity after 6 day incubation at 20 °C | 45*a* |

[a] Since native enzymes showed no activity in the IL, stability could not be determined.

The same research group recently reported the modification of cyt-*c* and P450st using PEO and hexylamine together to improve the solubility in ILs.[42] It was found that the modification of PEO with a long chain was effective for dissolution of the proteins while the structural changes in the vicinity of the heme group occurred by hexylamine modification.

We have shown that solubilization of subtilisin in ILs by chemical modification with comb-shaped poly-(ethylene glycol)($PM_{13}$)[43] (see Fig. 2) has also produced excellent catalytic activity and stability.[11c,44] The solubility and activity of modified enzymes were significantly dependent on the shape of PEG: enzymes modified with single-chained PEG were not soluble in [emim][$Tf_2N$] and showed very poor activity, whereas enzymes modified with double-chained PEG were soluble in IL but exhibited low activity.[44a] This is consistent with what was reported by Kaar *et al.* where linear-chain PEG-modified CRL did not show any activity.[10j] The average molecular weight of $PM_{13}$-modified subtilisin ($PM_{13}$-Sub) was measured by size-exclusion chromatography equipped with multi-angle light scattering (SEC-MALS) and shown to be ranging from 20 000 to around 1 000 000 Da.[44a] Despite the high molecular weight of $PM_{13}$-Sub, this formulation was readily solubilized in [emim][$Tf_2N$], [$C_2OC_1$mim][$Tf_2N$] and [$C_2$OHmim][$Tf_2N$], and exhibited extremely high activity, while native subtilisin showed no activity (see Table 4). Later, we modified CRL with $PM_{13}$ and found outstanding activity and stability for transesterification of 2-phenyl-1-propanol with vinyl acetate in ILs: $PM_{13}$-CRL in [emim][$Tf_2N$] maintained its original activity after 6 days.[45a] Another advantage of using these PEG-modified enzymes is that, since enzymes can be totally dissolved in ILs, the activities of the enzymes can be measured by spectrophotometric assay.[44b]

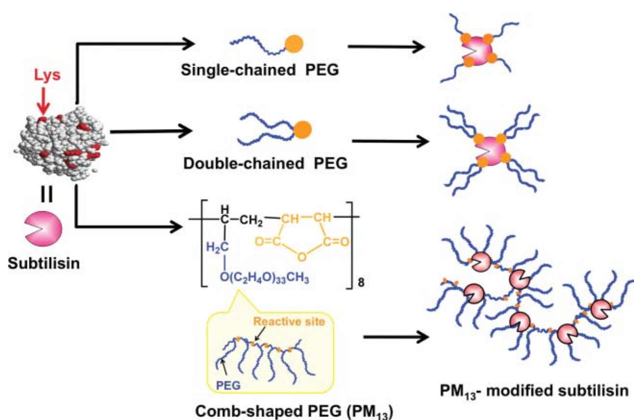


**Fig. 2** Schematic illustration of modification of subtilisin with PEG molecules.[11c,44a]

Ganske and Bornscheuer described the synthesis of glucose fatty acid esters in pure ILs using CaL-B immobilized on activated PEG.[23a] When commercial CaL-B was used as a biocatalyst, no conversion was observed in ILs. However, starting with CaL-B-PEG, conversions of 30 and 35% were obtained in [bmim][$BF_4$] and [bmim][$PF_6$], respectively, using lauric and myristic acid vinyl esters as the substrates. In another approach, the performance of penicillin G amidase (PGA) from *Escherichia coli* covalently immobilized on a polymer was investigated in different ILs.[45b] This enzyme preparation showed good synthetic activity (amide synthesis) and is fully stable in [bmim][$PF_6$] and [bmim][$BF_4$] at optimal water activity. However, no activity was found in hydrophilic [bmim][$CH_3OSO_3$] and [omim][$CH_3OSO_3$] due to the strong denaturing effects of the mesylate anion.

#### 3.1.4 Cross-linked enzyme aggregates (CLEAs).

The preparation of CLEAs, a new carrier-free immobilization technique invented by Sheldon *et al.*,[46] is a very useful approach to activate and stabilize many crude enzymes.[47] CLEAs are composed of almost entirely protein with just a small amount of cross-linking agent, and have advantages over CLECs (cross-linked enzyme crystals)[48] because the crystallization of the enzyme is often a lengthy procedure and requires high enzyme purity. Following from the pioneering work of Sheldon and co-workers on the use of CLEAs in ILs,[21a] a number of successful examples of the enzymatically active CLEAs have been reported.[49,50] Sheldon *et al.* [49a] have invented a CLEA of CaL-B (Novozyme 525F) which is active in ILs that deactivate the free forms of the CaL-B and other enzymes.[10j,k,51] The activity of CLEA CaL-B was further improved by the adsorption of CaL-B on a microporous polypropylene (CaL-B-PP).[49b] The CLEA CaL-B-PP was prepared by the addition of 2-propanol to lipase solution in the presence of microporous polypropylene to form aggregates of the enzyme, followed by glutaraldehyde cross-linking of lipase on polypropylene. CLEA CaL-B-PP gave much better transesterification activity in ILs and *tert*-butyl alcohol than did CLEA CAL-B (Table 5). The same enzyme preparations can catalyze the acylations of 1-phenylethanol and 1-phenylethylamine with high enantioselectivity in [bmim][$NO_3$] as well as in conventional organic solvents.

In a recent paper, Vafiadi *et al.*[49c] have shown that CLEAs can be made from feruloyl esterase (AnFaeA) on the addition of 100 mM glutaraldehyde, where ethanol was used as a precipitant. The CLEA produced was assayed for the enzymatic esterification of glycerol with sinapic acid, showing the first example of activity of FAEs in ILs. The synthesis of glycerol sinapate was found to be dependent on the IL cation, reaction temperature, water content and substrate concentrations. Although the catalytic activity of the

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**Table 5** Tranesterification of ethyl butanoate with 1-butanol in ILs using CLEA CaL-B[49b]

$$H_3C\text{-}CH_2CH_2\text{-}COOC_2H_5 \xrightarrow[\text{CAL-B, 40°C}]{n\text{-}C_4H_9OH/ILs} H_3C\text{-}CH_2CH_2\text{-}COOC_4H_9 + C_2H_5OH$$

| | Initial rate (μM min$^{-1}$ (g protein)$^{-1}$) | |
|---|---|---|
| Reaction medium | CLEA Cal-B | CLEA Cal-B-PP |
| [bmim][dca] | 6.6 | 228 |
| [bmim][$NO_3$] | 2.5 | 151 |
| [bmim][OAc] | n.d. | 62 |
| [bmim][$CH_3CH(OH)CO_2$] | n.d. | 58 |
| *tert*-Butyl alcohol | 220 | 374 |

CLEAs produced in this study was similar to the activity of the native enzymes, the immobilized AnFaeA was shown to maintain its activity over five consecutive 24 h period reaction cycles.

Shah and Gupta [50a] described the formulations of CLEAs using two enzymes, CRL and PCL, and have used them for kinetic resolution of (±)-1-phenylethanol in [bmim][$PF_6$] by transesterification with vinyl acetate. Both formulations showed better performance in terms of conversion and enantioselectivity than pH-tuned lyophilized lipase powder.[52] Particularly, CLEA PCL performed excellently: conversions after 2 h using CLEA and pH-tuned formulations were 41% ($E$ value > 1000) and 5% ($E$ value = 187), respectively. Very recently, Hara *et al.*[50b] employed CLEA PCL for the acylations of secondary alcohols in the most commonly used ILs (*e.g.*, [emim][$Tf_2N$], [emim][$BF_4$] and [bmim][$PF_6$]) and found that the modified lipase displayed higher initial rates than free PCL, as well as PCL immobilized by a sol–gel method, but loses its activity rapidly.

**3.1.5 Enzyme pretreatment with polar organic solvents.** The pretreatment of enzymes with polar organic solvents has been proven to be very effective in enhancing activity and enantioselectivity of enzymes in organic solvents. Among the various approaches, enzyme precipitated and rinsed with propanol (EPRP) has been used as an efficient method for preparation of highly active enzymes for synthesis in organic media.[53] This is a very simple one-step process for the preparation of free enzymes, using dry *n*-propanol (in some cases other lower alcohols or acetone) to precipitate the enzyme from a buffered solution, followed by washing with dry *n*-propanol (see Fig. 3). To date, a few studies of the enzymatic activity of EPRP in ILs have been reported,[37,50a] using CRL, BCL and subtilisin as biocatalysts. EPRP-treated subtilisin catalyzed the transesterification of APEE with *n*-propanol in [bmim][$PF_6$] with initial rates 4160 times greater than freeze-dried subtilisin powders.[37]

In another study, it was pointed out that EPRP-treated CRL and PCL could be used as very promising biocatalysts for the kinetic resolution of (±)-1-phenylethanol with vinyl acetate in [bmim][$PF_6$] (see Table 6).[50a] Note that the selection of solvents for precipitation as well as rinsing depends on the individual enzymes. For example, in the case of PCL, enzyme precipitated and rinsed with acetone (EPRA) was shown to be more suitable than EPRP.[50a] It has also been reported that PREP (propanol-rinsed enzyme preparations) gave higher reaction rates than freeze-dried powders in ILs. PREP involves drying of immobilized enzymes by rinsing with *n*-propanol instead of lyophilization which can cause structural changes in the enzyme molecule due to the removal

**Table 6** Examples of the improvement of enzyme performance in ILs when they are treated with organic solvents

| Enzyme | Preparations | Time | Conversion (%) | $E$ value | Ref. |
|---|---|---|---|---|---|
| PCL[a] | pH-tuned lyophilization | 2 | 8 | 187 | 50*a* |
| | EPRA | 2 | 49 | 736 | |
| | AREP | 2 | 50 | >1000 | |
| CRL[a] | pH-tuned lyophilization | 12 | 5 | 11 | 50*a* |
| | EPRP | 12 | 22 | 123 | |
| | PREP | 12 | 49 | 21 | |
| Subtilisin[b] | pH-tuned lyophilization | 8 | n.d. | — | 37 |
| | EPRP | 8 | 85 | — | |

[a] Kinetic resolution of (±)-1-phenylethanol by transesterification with vinyl acetate in IL [bmim][$PF_6$]. [b] Transesterification of *N*-acetyl-L-phenylalanine ethyl ester with *n*-propanol in IL [bmim][$PF_6$].

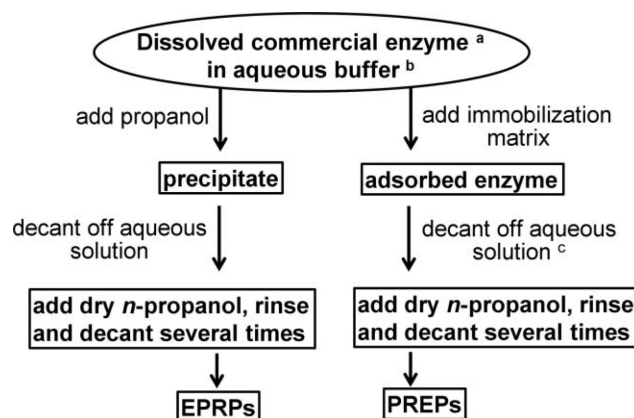


**Fig. 3** General procedure for the preparation of EPRPs and PREPs.[37,50a] [a] Highest purity needed; [b] pH of maximum activity; [c] No special treatment required.

of waters needed for maintenance of the enzyme's structure. Unlike, lyophilization, the propanol rinse rapidly removes water associated with protein in a way that minimizes denaturation and appears to leave the majority of enzyme molecules in an active conformation.[53a] This is a possible reason for these preparations exhibiting such excellent activity in non-aqueous ILs media.

### 3.2 Reaction media engineering

In addition to the enzyme formulations, various approaches to tailoring of the reaction media have been applied to maximize the activity of enzymes in IL systems. Three important strategies to modify IL media for biocatalytic reactions are discussed below.

**3.2.1 Water-in-ionic liquid microemulsions.** "Water-in-oil" (w/o) microemulsions, or reverse micelles, have been used as a very efficient technique for solubilizing enzymes in hydrophobic organic solvents while maintaining remarkably activity. Microemulsions are basically water droplets stabilized in non-polar solvents by a layer of surfactants, and are optically clear and thermodynamically stable.[54] Since the pioneering work of Luisi *et al.*,[55] w/o microemulsions have been used extensively as hosts for proteins and enzymes for carrying out biocatalytic reactions in organic solvents. The potential advantages of these microheterogeneous systems is that enzyme molecules are

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**Table 7** Biocatalysis conducted in w/IL microemulsions

| Surfactant | IL phase | Enzyme | Most important studies | Main results | Ref. |
|---|---|---|---|---|---|
| AOT | [omim][$Tf_2N$] | *Pseudomonas cepacia* lipase | Enzyme solubilization and activity studies | Better activity found than that in w/o microemulsions | 60 |
| Triton X-100 | [bmim][$PF_6$] | *P. chrysosporium* LiP, *T. versicolor* laccase | Investigation of activity as function of pH and $W_0$ | Activity of enzymes much higher than in pure/water saturated IL | 63 |
| Triton X-100 | [bmim][$PF_6$] | Yeast alcohol dehydrogenase (YADH) | IL inhibition effect on YADH studied, activity as function of pH and $W_0$ | YADH remains active but it has no activity in pure IL | 12*b* |
| AOT | [omim][$Tf_2N$] | Horseradish peroxidase (HRP) | Determination of $k_{cat}$, $K_m$ and stability | HRP intrinsic activity in w/IL microemulsions 5 times higher than in w/o microemulsions, stability comparable to w/o ME | 62 |
| Tween-20, Triton X-100 | [bmim][$PF_6$] | *C. rugosa* lipase, *C. viscosum* lipase, *T. lanuginose* lipase | Enzyme activity, stability and recycle studies, spectroscopic investigations | Excellent activity and stability, lipases reused 10 times without loss of catalytic activity | 64 |

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entrapped in tiny water domains, and thus are protected against unfavorable contact with the surrounding organic solvent by a layer of water and surfactant molecules, and thereby exhibit good stability and activity. In a similar manner, enzymes have been dissolved in compressed or supercritical carbon dioxide by the formation of water domains in $scCO_2$ (w/c microemulsions),[56] and the use of such microemulsions as "green" reaction media for enzymatic reactions is well-known.[57] Hopefully, in the last few years, a number of studies on the formation of water-in-IL microemulsions have been reported using anionic or non-ionic surfactants.[58,59]

In recent years, there have been a handful of publications regarding the enzymatic activity in water-in-ionic liquid (w/IL) microemulsions (see Table 7). We were the first to demonstrate that enzymes could be dissolved within the water pools of w/IL microemulsions using AOT (sodium bis(2-ethyl-1-hexyl)sulfosuccinate) as a surfactant and [omim][$Tf_2N$] as a continuous phase.[59] Subsequently, we reported the first example of an enzymatic reaction in w/IL microemulsions using lipase-catalyzed hydrolysis of *p*-nitrophenyl butyrate as a model reaction.[60] The fluorescence study of PCL encapsulated in the AOT/water/1-hexanol/IL system revealed that the lipase experiences an environment nearly identical to that of bulk buffered water, which contrasts with the AOT reverse micelles formed in a non-polar solvents.[60] This notable result was attributed to the large size of the water droplets formed in the IL microemulsions,[59] because Venables *et al.*[61] have shown that water in large microemulsions has a bulk-like character. Although PCL showed no significant activity in IL or even in water-saturated IL, the rate of enzyme-catalyzed reaction increased substantially when surfactant was added, allowing formation of water droplets in the IL.[60] More interestingly, the intrinsic activity of lipase in the IL microemulsion was about 3 times higher than that in microemulsions of AOT in isooctane. More recently, the use of w/IL microemulsions has been extended to activate and stabilize oxidoreductases, including HRP, lignin peroxidase (Lip), laccase and YADH (see Table 7).[12b,62] Zhou *et al.* reported that the apparent viscosity of the IL was decreased by forming the w/IL microemulsions,[63] which is beneficial for bioprocesses. Li and co-workers suggested that the inhibitory effect of IL on YADH can be overcome by separating the enzyme from IL using w/IL microemulsions.[12b]

More recently, three lipases from *Candida rugosa*, *Chromobacterium viscosum* and *Thermomyces lanuginosa* were successfully used in w/IL microemulsions with surfactants Tween-20 and Triton-100 in [bmim][$PF_6$] for lipase-catalyzed esterification.[64] The lipases exhibited superior catalytic performance and operational stability, particularly at higher incubation temperature (50 °C), than those observed in w/o microemulsions. For example, the half life of *C. viscosum* lipase in an aqueous solution, w/o and w/IL microemulsions at 50 °C was 0.9, 4.9 and 41.3 h, respectively. This stabilization was supported by Fourier transform-infrared (FTIR) and circular dichroism (CD) studies suggesting that in w/IL microemulsions, lipases retain their native structure or adopt more rigid structures than in other media.[65] The most notable finding of this study was the ease of product isolation and repeated use of enzymes, both of which are significant problems for the application of w/o microemulsions in industrial bioprocesses. The product and unreacted substrates could be extracted from the microemulsion simply by washing with *n*-hexane.[65] Lipase from *T. lanuginose* retained 90% of its activity after 10 reaction recycles in w/IL microemulsions formed with Tween-20 at 30 °C, indicating that the microenvironment of this microemulsion provides excellent protection to the entrapped enzymes even after repeated uses.

**3.2.2 Using organic solvents as (co)solvents.** Organic solvents have been used as (co)solvents/additives in IL reaction media to enhance the enzyme performance.[23a,66] Despite the promising results achieved with ILs in biocatalytic reactions, some important problems still need to be solved to further improve the enzyme activity. For example, the high viscosity of IL systems may limit the mass transfer of substrates and products to and from the active site of the enzyme, and in many cases can cause reduced conversion.[67] This problem tends to be more complicated when insoluble or sparingly soluble substrates and products are involved in IL media.[67b] In principal, high viscosity might be overcome by adding small amounts of organic solvents to IL systems. In addition, the solubility of hydrophobic substrates could be increased by using non-polar organic solvents as additives.

In 2005, Ganske and Bornscheuer applied a solvent system composed of [bmim][$BF_4$] and 40% *t*-butanol for the synthesis of glucose fatty acid esters using commercial CaL-B (Chirazyme L2 C2) (see Fig. 4).[23a] Using fatty acid vinyl esters as acyl donors, the conversions and isolated yields obtained were 90 and 89%, respectively. Similar encouraging results were reported by Chen *et al.*[66b] for the production of fatty acid ascorbyl esters with very

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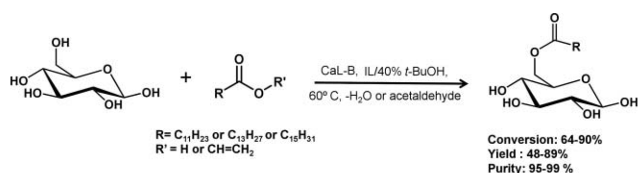


**Fig. 4** Production of glucose fatty acid esters in a two solvent system.[23a]

high volumetric productivity (120–200 g $L^{-1}$) in *t*-OMA·TFA/*t*-butanol systems (*t*-OMA·TFA = methyltrioctylammonium trifluoroacetate). In this system, the strong solvation of the IL allows the solubilization of a high concentration of ascorbic acid, and the presence of *t*-butanol increases the conversion by changing the equilibrium constant activity coefficients. Tan *et al.*[66d] used a mixture of [bmim][$PF_6$]–pyridine (80 : 20, v/v) for acylation of 1-β-D-arabinofuranosyl-cytosine using CaL-B as a catalyst and showed remarkable enhancement of substrate conversion compared with other solvent systems. The $a_w$ of the system was found to be crucial for this acylation reaction and under the optimal reaction conditions ($a_w$ = 0.11), the conversion and regioselectivity were 99.4 and 99%, respectively.

Banerjee *et al.* have employed IL–organic systems with lipases obtained from four different sources in different ILs in combination with various organic solvents (hexane, cyclohexane, isooctane, toluene, vinyl acetate and dichloromethane).[66a,c] Although it was observed that the reaction rates were highly dependent on the type as well as amount of ILs and organic solvents, in most cases IL/organic solvent systems exhibited promising results. For example, lipase from *P. aeruginosa* in [bmim][$PF_6$]–hexane (50 : 50 v/v) systems showed excellent stability at 70 °C and could be used repeatedly more than 10 times without significant loss of activity. In another report, Contesini and Carvalho demonstrated the performance of native lipases for enantioselective esterification of ibuprofen with 1-propanol in isooctane–IL systems, obtaining higher conversions to those in isooctane alone.[66e]

**3.2.3 Combination of ILs and $scCO_2$: A potential medium for biocatalysis.** The feasibility of using ILs as "green" solvents for biocatalysis is well-documented. However, a second phase is required for recovery of the products and any remaining reactants. Typically, volatile organic solvents (VOSs) have been used to separate the products from ILs, which reduced the "green" aspect of the systems. In addition, in many previous studies, VOSs have been used to reduce the viscosity of IL media as discussed in the above section. Encouragingly, in 1999, Brennecke and co-workers[39] discovered that supercritical $CO_2$ ($scCO_2$) can be used as an environmentally benign solvent to extract organic products or contaminants from ILs. Following this work, plenty of articles have been published associated with examining the solubility of $scCO_2$ in various ILs and the phase behavior of IL–$CO_2$–solute ternary systems.[68] Applying these methodologies, a number of groups have studied IL/$scCO_2$ systems for biocatalytic reactions.[36a,69-71] In these systems, the biocatalyst is retained in an IL working phase, while the reactants and products largely reside in a $scCO_2$ extractive phase. Consequently, the IL-enzyme phase can easily be recycled and reused for other batches, and the product can be recovered from the $scCO_2$ phase by simply decreasing the pressure. In most cases, the activity and stability of enzymes were found to be higher in IL/$scCO_2$ systems than in the IL alone.

In 2001, Laszlo and Compton described the use of an IL/$scCO_2$ system for the transesterification of APEE with 1-propanol using α-chymotrypsin as a model biocatalyst, and suggested the use of $scCO_2$ to separate the product from the IL.[36a] They used two ILs, [bmim][$PF_6$] and [omim][$PF_6$] in which $scCO_2$ is highly soluble. In a later study, Lozano *et al.*[69a] presented the results of two reactions (transesterification of vinyl butyrate with 1-butanol and the kinetic resolution (KR) of *rac*-1-phenylethanol with vinyl propionate) in [emim][$Tf_2N$]/$scCO_2$ and [bmim][$Tf_2N$]/$scCO_2$ systems catalyzed by lipase CaL-B. In both ILs, the enantiomeric excess of the recovered product fraction ($ee_p$) for continuous (*R*)-1-phenylethyl propionate synthesis at 100 °C and 15 MPa was above 99.9%, and the enzyme showed good activity and stability. The same group has tested the catalytic activity and stability of both free CaL-B (NZ 525 L) and immobilized CaL-B (NZ 435) in IL/$scCO_2$ systems composed of [emim][$Tf_2N$] and [bmim][$Tf_2N$] for the continuous KR of *rac*-1-phenylethanol.[69b] The results showed that the enzyme shows excellent activity and stability under extremely denaturing conditions (*e.g.*, 150 °C and 10 MPa). Recently, Lozano *et al.* described for the first time the continuous dynamic KR of *rac*-1-phenylethanol in IL/$scCO_2$ biphasic systems, using a combination of the immobilized CaL-B(NZ 435) and silica modified with benzenesulfonic acid (SCX) as catalysts. Coating both chemical and enzymatic catalysts with ILs greatly improved the efficiency of the process, obtaining a good yield (76%) of (*R*)-1-phenylethyl propionate product with excellent enantioselectivity (ee = 97.4%) in continuous operation at 40 °C and 100 MPa.[69c] However, VOS (*e.g.*, a hexane solution) addition was required to introduce the starting ester into $scCO_2$, which certainly decreases the impact of using ILs as alternatives for VOSs.

To overcome this problem, Leitner and co-workers reported a new approach for enzymatic reactions in ILs using $scCO_2$ as the mobile phase.[70] They tested the activity of CaL-B for the acylation of 1-octanol with vinyl acetate in [bmim][$Tf_2N$]/$scCO_2$ biphasic systems in which substrates were added directly to the IL–enzyme phase rather than employing a carrier solvent.[70a] In this case, the $scCO_2$ was used as an extraction solvent since it was added only after 0.5 h to extract the product and unreacted reactants from the system for collection in a cold trap after evaporation of the $CO_2$. The IL phase containing the CaL-B was recycled three times with yields of 97, 98, and 98%, respectively, suggesting that the enzyme retains good activity. The same system was then modified into a continuous process with $scCO_2$, producing 0.1 kg of *n*-octyl acetate per litre of reactor volume per hour. The process was continued over a 24 h period, giving an overall yield of 93.9%. A batchwise operation was also found to be very effective for KR of *rac*-1-phenylethanol with vinyl acetate. The enantioselectivity of the reaction was found to be greater than 98.6% for the (*R*)-acetate over four cycles.[70a] In a later study, the KR of secondary alcohols was transformed from a batch to a continuous process, and the systems were optimized using various ILs, esters, and enzyme preparations.[70b] Reaction with vinyl laureate gave an ester that had much lower solubility in $scCO_2$ than the unreacted *rac*-1-phenylethanol, facilitating its downstream separation by controlled density reduction of the $scCO_2$ *via* a change in temperature and/or pressure. Results obtained from the use of sol–gel-immobilized CaL-B instead

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of the suspended lyophilized CaL-B suggested that this enzyme formulation gave considerably lower yields with vinyl acetate as the acylating species.

Recently, Bogel-Łukasik *et al.*[71] presented the vapor–liquid equilibrium (VLE) data for a ternary system composed of IL/$scCO_2$/products of lipase-catalyzed acylation of (*R*,*S*)-2-octanol with succinic anhydride. The choice of IL and experimental conditions were examined. The extraction study showed that an [omim][PF6]/scCO2 system at 35 °C and 11 MPa allowed recovery of >99.99 mol% of unreacted (*S*)-2-octanol with low coextraction of other solutes, as well as giving a very high enantiomeric excess (98.42%). In another study, the catalytic activity of NZ 435 and *Fusarium solani pisi* cutinase immobilized on zeolite NaY in a [bmim][$PF_6$]/$scCO_2$ system in continuous operation at 35 °C and 10 MPa was investigated for the reaction of 2-phenyl-1-propanol and vinyl butyrate.[20c] For both enzymes, the rate observed in the IL/$scCO_2$ system was found to be higher than in IL alone, and this was attributed to the $scCO_2$ reducing the viscosity of the IL and causing favorable mass transfer of substrates to the enzyme active site. However, the enantioselectivity of both enzymes for the substrate was found to be low ($E = 1.5$–3).

## 4. Ionic liquid–enzyme complexes: A new concept for biocatalyst preparation

In parallel to the use of ILs as solvents for enzymes, ILs can be used as immobilizing/coating agents for enzymes. While this topic is outside the subject of this review, it deserves to be mentioned as an emerging technique for the formation of biocatalysts that could exhibit better catalytic activities, stabilities and enantioselectivities in harsh reaction conditions. In this approach, Liu and co-workers immobilized CRL on functionalized ILs (see Fig. 5) which were obtained by covalent bonding of ILs to silane on magnetic silica nanoparticles.[72] The immobilized lipase catalyzed the esterification of oleic acid with butanol in a solvent-free system with better activity, thermostability and reusability than those observed for the free enzyme: immobilized lipase retained 60% of its initial activity after 8 repeated batch reactions, while no activity was detected after 6 cycles for the free enzyme.

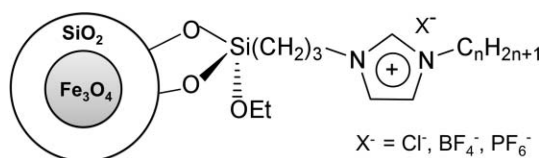


**Fig. 5** Functionalized ionic liquids stabilized $Fe_3O_4$ nanoparticles used for enzyme immobilization.[72]

In recent years, the coating of enzymes with ILs has emerged as an efficient method for the preparation of highly active and stable biocatalysts for non-aqueous biotransformation.[73–75] To date, various techniques with different IL coating agents have been used for immobilization of enzymes. In most cases IL-coated enzymes displayed better catalytic activities, stabilities and/or enantioselectivities in organic solvents and/or solvent-free conditions compared to free enzymes. For example, PCL coated with [ppmim][$PF_6$] (ppmim = 1-(3′-phenylpropyl)-3-methyl-imidazolium) showed markedly higher enantioselectivities for the kinetic resolution of vinyl acetate with various racemic secondary alcohols in toluene.[73a] In a study by Itoh *et al.*,[74b] PCL and CRL were coated with [bdim][cetyl-PEG-10-sulfate] (bdim = 1-butyl-2,3-dimethylimidazolium). The catalytic activity was assayed for acetylation of various types of secondary alcohols using vinyl acetate as an acyl donor in *i*-propanol with more than a 1000-fold rate acceleration for some substrates. MALDI-TOF mass spectrometric analysis indicated that IL binds to the enzymes, and thus provides a favorable environment for the reaction. Very significantly, CaL-B immobilized with [bmim][$PF_6$] was active at very high temperature (95 °C) in hexane and solvent-free conditions.[75a] On the other hand, Rumbau *et al.* have shown that HRP immobilized in [bmim][$PF_6$] acts as an efficient biocatalyst for synthesis of polyaniline. This method was found to be faster and easier than the classical immobilization of HRP on solid supports.[18d]

Very recently, our group has reported the encapsulation of HRP in polymerized IL microparticles (pIL-MPs), which were prepared by polymerization of 1-vinyl-3-ethylimidazolium bromide [veim][Br] in the presence of the cross-linker *N*,*N*′-methylenebis (acrylamide) in a concentrated w/o emulsion, as shown in Fig. 6.[76] The pIL-MPs containing PEG-modified HRP exhibited excellent activity for guaiacol oxidation in an aqueous solution. The activity of HRP incorporated in pIL-MPs was much higher than that of the enzyme encapsulated in polyacrylamide microparticles. This technique enables the biocatalyst to be easily recycled simply by centrifugation for use in repeated reactions.[76]

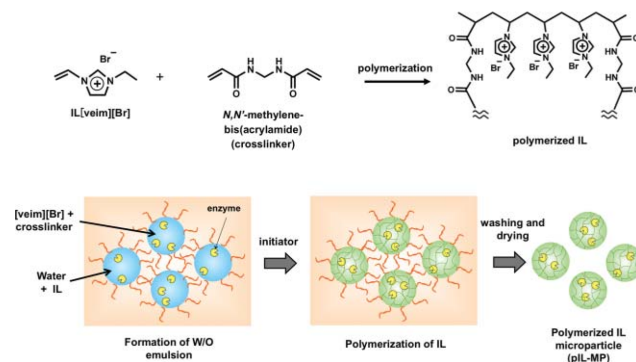


**Fig. 6** Production of polymerized microparticles containing enzyme.[76]

## 5. Conclusions and outlook

This review brings together a number of techniques that could be used for activation and stabilization of enzymes in ILs, which are receiving increasing attention as "green" solvents for organic synthesis and catalytic processes. Many enzyme formulations show exciting potential for carrying out biocatalytic reactions in ILs, suggesting a promising future for modified enzymes in these new non-aqueous solvent systems. The enzyme formulation is particularly important when hydrophilic ILs are used as the reaction media. However, for most formulations, additional studies are required to obtain quantitative and physically robust data to design optimal enzyme formulations for use in IL media. We believe that the combination of these preliminary studies will provide clear routes to produce activated as well as stabilized enzyme formulations for use in ILs. An increasing amount of

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research in this field can be expected in the coming years. Furthermore, the utility of the biocatalysis in ILs would be markedly improved if various enzyme formulations with a broader assortment of enzymes were commercially available at reasonable prices. The main drawback of using ILs is the significant uncertainty regarding the toxicity and potential impact of ILs on the environment. However, in both of these areas, significant steps have been made in the understanding and creation of cleaner routes to the preparation of ILs.[77] We believe that green and biocompatible ILs will be available in the near future, which will stimulate the use of ILs in industrial biotransformations.

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## Notes and references


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