Lipase dissolution and stabilization in ether-functionalized ionic liquids†

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Ionic liquids (ILs) with various structures and properties have been extensively investigated in many biocatalytic reactions and processes. However, although hydrophobic ILs tend to stabilize the insoluble (suspended) enzymes, they usually have low hydrogen-bonding basicity with solutes, which limits the solubility of many substrates (such as D-glucose, ascorbic acid, and cellulose). In contrast, hydrophilic ILs (such as those based on chloride, acetate and dicyanamide) are able to dissolve many of these substances that are not quite soluble in common organic solvents. Unfortunately, enzymes are not always active in these hydrophilic media due to strong interactions (such as H-bonding) between proteins and ILs. To resolve this dilemma, we recently synthesized new acetate-based ILs carrying a long alkyloxyalkyl chain in their cations, and found that these ether-functionalized solvents are lipase-compatible and can dissolve considerable amounts of D-glucose and cellulose (*Green Chem.*, 2008, **10**, 696). In this study, we further observed that these ILs could dissolve high concentrations of lipase B from *Candida antarctica* (CALB) (> 5 mg/mL at 50 °C), as well as other substrates including amino acids and betulinic acid. Therefore, these novel media offer new opportunities for carrying out homogeneous enzymatic reactions, which is practically important for large substrate molecules. In this article, we further confirmed the lipase compatibility of these ILs through the transesterification between ethyl butyrate and 1-butanol. The second derivative infrared spectra of CALB suggest the conservation of secondary structures of proteins in these ILs. We further investigated these ether-functionalized ILs in two important biocatalytic reactions: enzymatic synthesis of methyl-phthalate of betulinic acid, and CALB-catalyzed synthesis of D-glucose fatty acid esters. These substrates are not very soluble in conventional organic solvents, but very soluble in ILs, which improved the catalytic efficiency of these reactions. Moderate to high conversions were achieved in both reactions.

Introduction

Biocatalysis in organic solvents undoubtedly offers numerous advantages and intriguing opportunities over aqueous media.[1,2] However, even with the modifications of enzymes and solvent systems, enzymes are typically less active in organic media than in water.[3,4] In particular, when the substrate molecules are sparingly soluble in organic solvents, the biotransformation becomes extremely challenging since enzymes are also insoluble in low-water systems. To overcome this hurdle, a common strategy is to solubilize the enzyme in organic media by either chemical modifications or the formation of reversed micelle of enzymes, but both methods have some serious disadvantages.[5] The Dordick group[6,7] reported a method in dissolving enzymes in organic solvents with low concentrations of surfactants. Based on this technique, the transesterifications of solid β-cyclodextrin, amylose, hydroxy ethyl cellulose, and cellulose were conducted in isooctane or pyridine using soluble subtilisin Carslberg.[5,8,9] However, these substrates were still suspended in solutions as heterogeneous systems. Therefore, an ideal solution would be a solvent system that can dissolve both the enzyme and substrates, while the enzyme maintains a high activity in the solvent.

As alternatives to conventional organic solvents, ionic liquids (ILs) belong to a young family of non-aqueous solvents. In addition to their low-volatility, ILs have tunable physical properties through the judicious selection and combination of different cations and anions. Therefore, ILs have found a wide range of applications in organic reactions,[10] biocatalysis,[11] chemical processes,[12] and other areas.[12,13] Pure hydrophobic ILs (typically consisting of PF_6^- and Tf_2N^-) do not dissolve appreciable amounts of enzymes; on the other hand, hydrophilic ILs (such as those based on NO_3^-, lactate, $EtSO_4^-$, and CH_3COO^-) and their aqueous solutions may dissolve some enzymes,‡ however, most of them tend to strongly interact with proteins (such as *via* hydrogen bonding), resulting in enzyme inactivation.[14–20] At present, only a few enzyme-dissolving ILs are known enzyme-compatible. For example, choline dihydrogen phosphate (m.p. 119°C) containing 20% (wt) water was observed capable of dissolving and stabilizing cytochrome *c* (cyt *c*).[21,22] Another IL,

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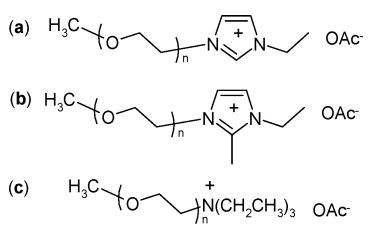
‡ There are also some exceptions. For example, BF_4^- based ILs are hydrophilic but do not dissolve the enzyme.[14]

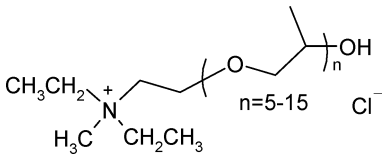
triethylmethylammonium methyl sulfate ([Et_3MeN][$MeSO_4$])§ was reported able to dissolve > 1.2 mg/mL *Candida antarctica* lipase B (CALB) and retain its catalytic capability.[14,23] Thus, to enable the homogeneous biocatalysis in ILs, a common route is to modify the enzyme molecules with crown ether or poly(ethylene glycol) (PEG), both of which are soluble in ILs.[14,24,25] However, the preparation of PEG-modified enzymes could be cumbersome, and the enzyme catalytic properties may vary in different immobilization batches.

Very recently, we synthesized a new type of acetate-based ILs (Scheme 1) through crafting oligoethylene (or oligopropylene) glycol units into the cations; these ILs exhibit a number of favorable properties including high lipase compatibility, low viscosity, and 'super' solvents for a variety of substrates such as cellulose and sugars.[15] In addition, we noticed that these ILs could dissolve a considerable amount of enzymes (data in a later section). Therefore, these ether-functionalized ILs have great potentials for homogeneous enzymatic reactions in ILs. Historically, it has been known that poly(ethylene oxide)s (PEOs) can be incorporated into cationic or anionic units to produce the liquid state of ion conductive polymers.[13,26] In particular, the liquid state of ionic polymers has been synthesized by grafting alkyloxy substituents (ether or alcohol groups) onto the imidazolium ring.[27] Following these studies, ether-functionalized ILs based on imidazolium[28–37] and pyridinium[38] have been synthesized and their physical properties were determined. The inclusion of alkyloxy or alkyloxyalkyl groups lowers the melting-points and viscosities of the organic salts, resulting in room-temperature ILs in most cases. The low viscosity resulting from the incorporation of alkoxy chains was justified by the molecular dynamic simulations as the less effective assembly between more flexible alkoxy chains (*vs.* more rigid alkyl chains).[39] In addition, the molecular simulation also suggested the reduction of intermolecular correlation (particularly tail-tail segregation) and cation-anion specific interactions due to the incorporation of ether chain, which is responsible for the faster dynamics in ether-functionalized imidazolium ILs compared to alkyl substituted ILs.[40]



Scheme 1 Imidazolium and ammonium-based ILs consisting of alkyloxyalkyl-substituted cation and acetate anion ((**a**) [$Me(OEt)_n$-Et-Im][OAc], (**b**) [$Me(OEt)_n$-Me-Et-Im][OAc], and (**c**) [$Me(OEt)_n$-Et_3N][OAc] respectively) (n = 2, 3, . . .).

A few groups have reported the uses of these ether-functionalized ILs in the enzyme stabilization. 'Ammoeng 110' is a commercial quaternary ammonium mixture containing oligopropylene glycol units (Scheme 2). Our previous investigations



Scheme 2 Structure of AMMOENG™ 110 ([Amm110]Cl).

suggested that the immobilized CALB retained high synthetic activities in this IL.[15,20] The same IL was also used to form aqueous biphasic systems for the biocompatible purification of active enzymes.[41] Other similar ammonium ILs in the Ammoeng family carrying alkyloxyalkyl groups have also been used in stabilizing lipases for the glycerolysis of fats and oils.[42,43]

Although our previous study has suggested the high enzyme-compatibility of immobilized CALB (Novozym® 435) in ether-functionalized ILs,[15] we have not fully understood the underlying mechanisms *via* the free enzyme. It is the aim of this study to examine the dissolution of lipase in these promising ILs, to investigate the catalytic capability of free/immobilized lipase in these media, and to understand how IL structures affecting the enzyme stabilization. We also demonstrate the application of these ether-functionalized ILs in two important reactions: enzymatic synthesis of methyl-phthalate of betulinic acid, and CALB-catalyzed synthesis of D-glucose fatty acid esters.

Experimental

General

The following chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA): ethyl butyrate, butyl butyrate, 1-butanol, 1-ethyl-3-methylimidazolium bromide ([EMIM]Br), 1-butyl-3-methylimidazolium bromide ([BMIM]Br), 1-methyl-3-octylimidazolium bromide ([OMIM]Br), 1-ethyl-3-methyl-imidazolium ethylsulfate ([EMIM][$EtSO_4$]), 1,3-dimethylimidazolium methylsulfate ([MMIM][$MeSO_4$]), 2-methylimidazole, bis(trifluoromethane)sulfonimide lithium salt (Li[Tf_2N]), sodium dicyanamide (Na[dca]), sodium acetate, phosphorus pentoxide (P_2O_5), L-ascorbic acid, vinyl laurate, D-glucose, $^{13}C_6$-labeled D-glucose, betulinic acid, dimethyl phthalate and glucose HK assay. 1-Ethyl-3-methylimidazolium trifluoromethanesulfonate ([EMIM][OTf] or [EMIM][CF_3SO_3]) was obtained from the Alfa Aesar Company (Ward Hill, MA, USA).

AMMOENG™ 110 (short as [Amm110]Cl, Scheme 2) was obtained from Solvent Innovation GmbH (Nattermannallee, Germany) as a colorless liquid (mp < −65 °C, density = 1.03 g/cm^3 at 20 °C, viscosity = 495 mPa s at 20 °C, pH = 3.83 and conductivity = 0.090 mS/cm).

The free form lipase B from *Candida antarctica* (CALB) was also obtained from Sigma-Aldrich (product # 62288, recombinant from *Aspergillus oryzae*, powder, beige, ~9 units/mg). Novozym® 435 from Sigma-Aldrich is a thermal stable CALB immobilized on acrylic resin (product # L4777, ≥10,000 U/g, recombinant, expressed in *Aspergillus oryzae*).

IL preparations

The preparation of [BMIM][Tf_2N], [BMIM][dca], [BMIM][HCOO], [EMIM][OAc] and [OMIM][OAc], as

§ This IL was reported as a liquid at room temperature,[14] however, it tends to form liquid/solid co-existing state at room temperature and 50 °C (see Experimental).

well as the alkyloxyalkyl substituted ILs (Scheme 1) was described in our recent paper.[15] All ILs were dried in an oven at 100 °C over 4 h before use. Acros® 3A molecular sieves were added into ILs during storage. The characterization of these ILs were reported previously[15] except three new ILs (see ESI† for NMR characterization of [$Me(OEt)_3$-Et_3N][HCOO], [$Me(OEt)_3$-Me-Et-Im][OAc] and [$Me(OEt)_3$-Et-Im][dca]).

The preparation of triethylmethylammonium methylsulfate ([Et_3MeN][$MeSO_4$]) followed a literature method.[14] The purified product became semi-solid after cooling to room temperature (and at 50 °C). ^{1}H NMR (300 MHz, $CDCl_3$, [ppm]): δ = 1.37 (t, 9H, CH_2CH_3, J = 2.1 Hz), 3.06 (s, 3H, SO_4CH_3), 3.43 (m, 6H, CH_2CH_3), 3.68 (s, 3H, NCH_3).

Measuring CALB transesterification activity

Organic solvents were dried by anhydrous $MgSO_4$ before use. The free CALB and Novozym 435 were dried over P_2O_5 for at least 2 days. A typical reaction procedure is as the following: 14 μL ethyl butyrate (0.1 M) and 46 μL 1-butanol (0.5 M) were added into a glass reactor containing organic solvent or IL. The final mixture volume was 1.0 mL. The reaction was started by adding 5.0 mg free CALB, or 25 mg Novozym 435. The reaction mixture was sealed and stirred at 50 °C in a water-bath. The reaction mixture was periodically withdrawn (50 μL each sample) and diluted with 100 μL methanol. After centrifugation, the clear supernatant was injected into a LC-10AT Schimadzu HPLC equipped with a SPD-10Avp UV-visible dual wavelength detector and a Schimadzu RID10 refractive index detector. The injection loop volume was 20 μL. The HPLC eluent consisted of 65% (v/v) MeOH and 35% (v/v) aqueous acetate buffer (0.05 M, pH 4.5). The flow rate was 1.0 mL min^{-1}. The column is a Schimadzu Premier C18 column (150 mm × 4.6 mm, particle size 5 μ). The UV detection wavelength was 215 nm. The product concentration was determined by comparing the sample's peak area with the standard curve of butyl butyrate. All experiments were run at least in duplicate. The percent errors were less than 5%.

CALB stability in ILs

Novozym 435 (25 mg, dried over P_2O_5) was incubated in 1.0 mL IL under a gentle agitation at 50 °C. After certain incubation time, 14 μL ethyl butyrate (0.1 M) and 46 μL 1-butanol (0.5 M) were added to the enzyme suspension in IL. The reaction was maintained at 50 °C for 24 h, and the product concentration was determined by the HPLC analysis (see above).

FT-IR spectra of CALB in ILs

Free CALB was dissolved in ILs (2 mg/mL) or water (10 mg/mL) and incubated in water bath at 50 °C. The enzyme solution was taken periodically and placed between two CaF_2 windows. The FT-IR spectra were measured using a Shimadzu IRPrestige-21 spectrophotometer through averaging 32 scans at 2 cm^{-1} resolution (Happ-Genzel apodization). The respective pure IL or water between CaF_2 windows was scanned as the background before each measurement. The original spectra were smoothed based on the 9-point Savitsky-Golay algorithm. The second derivatives of spectra were calculated *via* the Shimadzu IRsolution 1.30 software following the Savitsky-Golay method (9 data point window).

Enzymatic synthesis of methyl-phthalate of betulinic acid

Betulinic acid (52 mg, 0.11 mmol) and dimethyl phthalate (25.8 mg, 0.13 mmol) were fully dissolved in 1.0 mL [$Me(OEt)_3$-Et-Im][OAc] (**3**) after a gentle agitation. Novozym 435 (40 mg, undried by P_2O_5) was added into the reaction mixture in a capped glass-vial reactor. The reaction was kept at 50 °C. Upon completion of reaction, the reaction mixture was diluted with 10 mL methanol and the immobilized enzyme was removed through filtration. After evaporation of methanol under reduced pressure, the crude product was dissolved in 200 mL ethyl acetate, and washed with distilled water three times to remove the IL and trace dimethyl phthalate (slightly soluble in water). The product was collected after drying the ethyl acetate with sodium sulfate, filtering off the salt, and evaporating the solvent under reduced pressure. The isolated product weighed 60 mg (yield 85%). IR ν (KBr) cm^{-1}: 3475, 2949, 2870, 1732, 1687, 1450, 1435, 1125, 1076. ^{1}H NMR (JEOL ECX-300 MHz, DMSO-d_6, [ppm]): 0.61 (3H, s), 0.72 (3H, s), 0.83 (3H, s), 0.89 (3H, s), 1.61 (3H, s), 3.28 (3H, s), 3.78 (3H, s), 4.20 (3H, m), 4.55 (3H, m), 4.66 (3H, m), 7.65 (4H, m), 12.05 (1H, s). ^{13}C NMR (DMSO-d_6) showed two new signals for the product at 129.2 ppm and 132.2 ppm, indicating the incorporation of two ester carbons (–COOR) from phthalate.

Lipase-catalyzed synthesis of D-glucose fatty acid ester

D-Glucose (0.0396 g, 0.22 mmol) and vinyl laurate (0.075 g, 0.33 mmol) were added into 1.0 mL IL in a capped glass-vial reactor. After both substrates fully dissolved in the IL under a gentle stirring at 50 °C, Novozym 435 (40 mg, dried over P_2O_5) was carefully added into the mixture. The reaction was kept at 50 °C and monitored over 30 h. Periodically, 30 μL reaction mixture was withdrawn and mixed with 970 μL distilled water. After centrifugation (or filtration) to settle (or remove) the precipitated acid and ester, the glucose concentration in supernatant (or filtrate) was determined by the glucose HK assay.[44] Upon completion of reaction, the reaction mixture was diluted with 10 mL methanol and the immobilized enzyme was removed through filtration. After evaporation of methanol under reduced pressure, the crude product was precipitated from IL by the addition of distilled water. The crude product was collected into chloroform[45] (or dichloromethane) and washed several times with water to remove IL. The glucose fatty acid ester was purified through washing with hexane to remove the remaining fatty acid.[46] After drying the chloroform, the solvent was removed under vacuum. The structures of product (see ESI†) were confirmed by ^{1}H and ^{13}C NMR ($^{13}C_6$-labeled D-glucose was used as reactant for the analysis of ^{13}C NMR of product).

Results and discussion

Selection of assay for measuring lipase's synthetic activity

A number of transesterification reactions are routinely carried out to evaluate the enzyme's synthetic activity in non-aqueous

systems. It is very common to use vinyl ester as acyl donor because the transesterification between vinyl ester and an alcohol is irreversible and fast, although it is known that the by-product acetaldehyde may lead to some degree of enzyme inactivation.[47,48] Therefore, the enzyme-catalyzed acyl transfer reactions have been frequently examined in ILs.[11,49] However, when conducting the lipase-catalyzed transesterification between vinyl butyrate and benzyl alcohol in formate- and acetate-based ILs (*i.e.* [Bu_4N][HCOO] (**2**), [$Me(OEt)_3$-Et-Im][OAc] (**3**), and [$Me(OEt)_3$-Et_3N][OAc] (**4**)), we observed comparably fast reaction rates even in the absence of CALB (see Table 1). The reaction did not proceed in [BMIM][Tf_2N] (**1**), [BMIM][dca] (**6**), [EMIM][$EtSO_4$] (**9**), and [MMIM][$MeSO_4$] in the absence of enzyme. This observation suggests that formate- and acetate-type ILs can catalyze the acylation reaction since the vinyl ester is an activated substrate and the reaction is irreversible. For this reason, the transesterification reaction involving vinyl ester is not suitable for evaluating the lipase's synthetic activity in formate- or acetate-based ILs. In fact, the acetate-catalyzed transesterification (or esterification) was used in the industrial production of PET (polyethylene terephthalate), where dimethyl terephthalate (or tetrephthalic acid) reacts with an excess of ethylene alcohol catalyzed by Cu, Co, or Zn acetate at 100–150 °C and 10–70 bar.[50] It is also known that ILs based on acetate, formate and dicyanamide are Lewis bases with various hydrogen-bonding basicity (proton acceptors), and may act as base catalysts in reactions such as acetylation of alcohols.[51,52]

Alternatively, another transesterification reaction between ethyl butyrate and 1-butanol was chosen because no reaction was detected in formate- and acetate-based ILs (**2–4**) without CALB. This reaction is relatively fast when catalyzed by immobilized CALB, and can reach equilibrium within a few hours at 40 °C[20,53] or 100 °C.[54] However, the same reaction catalyzed by free CALB is much slower, and it usually takes 24 h to reach completion at 40 °C[14,23,53] or 100 °C.[55] It is known that the immobilized lipases usually induce higher reaction rates than free enzyme particles in organic media.[56,57] The same improvement effect was seen with other enzymes such as proteases, thermolysin, liver alcohol dehydrogenase, mushroom polyphenol oxidase and carboxyesterase.[57,58] Thus, we conducted this model reaction in *t*-butanol and a few ILs catalyzed by both free and immobilized CALB (Table 2). Our data confirm that the reaction rates were higher in all ILs when using the immobilized CALB instead of free lipase. However, most reactions in ILs with immobilized CALB still took 24 h to complete, although most of the product (butyl butyrate) was formed within the first 5 h. Based on the estimation that Novozym 435 contains ~20% (wt) of CALB,[59] the protein

Table 1 Initial rate (μmol/min mg) of transesterification between vinyl butyrate and benzyl alcohol at 50 °C [a,b]

	IL	Free CALB	Control (no enzyme)
1	[BMIM][Tf_2N]	0.21	0
2	[Bu_4N][HCOO]	1.25	1.23
3	[$Me(OEt)_3$-Et-Im][OAc]	0.89	0.54
4	[$Me(OEt)_3$-Et_3N][OAc]	0.90	0.88

[a] Reaction conditions: 0.1 M vinyl butyrate (13 μL), 0.5 M benzyl alcohol (103 μL), 2.0 mg free CALB or no enzyme (as control) in 1.0 mL IL at 50 °C. [b] HPLC conditions: 65% (v/v) acetonitrile and 35% (v/v) aqueous acetate buffer (0.05 M, pH 4.5); both vinyl butyrate and benzyl butyrate were detected at 215 nm.

Table 2 CALB activity in ILs at 50 °C (percent yield of butyl butyrate) [a]

Solvent		Immobilized CALB		Free CALB		Solubility of free CALB (mg/mL) [d]	[Anion] (M) [e]
		5 h	24 h	5 h	24 h		
5	t-BuOH	70	73	11	26	— [b]	—
1	[BMIM][Tf_2N]	62	62	35	60	— [b]	2.86
6	[BMIM][dca]	33	48	4	17	5	5.85
7	[EMIM][OAc]	17	44	11	22	5	7.05
8	[OMIM][OAc]	56	70	11	40	5	4.72
9	[EMIM][$EtSO_4$]	57	86	38	67	2	5.08
2	[Bu_4N][HCOO]	— [c]	41	23	25	2	4.17
2a	[BMIM][HCOO]	33	15	4	14	5	6.52
10	[Amm110]Cl	43	51	37	54	1	—
13	[$Me(OEt)_2$-Et-Im][OAc]	60	56	6	17	> 5	4.65
3	[$Me(OEt)_3$-Et-Im][OAc]	42	61	21	46	> 5	3.97
14	[$Me(OEt)_3$-Bu-Im][OAc]	55	81	6	18	4	3.63
15	[$Me(OEt)_7$-Et-Im][OAc]	58	78	14	43	1	2.46
16	[$Me(OEt)_3$-MeOEtOMe-Im][OAc]	42	48	13	26	1	3.31
17	[$Me(OPr)_3$-Et-Im][OAc]	57	72	8	21	> 5	3.48
18	[$Me(OEt)_3$-Me-Et-Im][OAc]	34	62	5	22	1	3.79
19	[$Me(OEt)_3$-Et-Im][dca] [f]	32	40	38	55	5	3.88
11	[$Me(OEt)_2$-Et_3N][OAc]	25	36	10	31	4	4.56
4	[$Me(OEt)_3$-Et_3N][OAc]	41	51	17	46	> 5	3.90
12	[$Me(OEt)_3$-Et_3N][HCOO]	37	65	12	35	4	4.09
20	[EMIM][OTf]	30	33	3	20	— [b]	4.61

[a] Reaction conditions: 0.1 M ethyl butyrate, 0.5 M 1-butanol, 5.0 mg free CALB or 25 mg Novozym in 1.0 mL solvent at 50 °C. [b] solubility is too low to be observed. [c] The HPLC yield was not obtained due to difficulty in integration. [d] The solubility was determined by an incremental addition of 0.5 mg CALB into 1.0 mL IL. [e] The anion molar concentration was calculated from eqn 1; Ammoeng 110 is an IL mixture and its molecular weight is unknown; [f] A low viscosity (50 mPa s) was obtained for this dca-based IL (using method in Ref. 15).

contents in 25 mg Novozym 435 and 5 mg free CALB are about the same.

Lipase dissolution and stabilization in ILs

The lipase solubility data in Table 2 suggest that the CALB is not quite soluble in *t*-butanol (**5**) and hydrophobic ILs such as [BMIM][Tf_2N] (**1**).¶ The lipase has a low solubility (1 mg/mL or less) in [Amm110]Cl (**10**), [EMIM][OTf] (**20**), [Me(OEt)$_7$-Et-Im][OAc] (**15**), [Me(OEt)$_3$-MeOEtOMe-Im][OAc] (**16**), and [Me(OEt)$_3$-Me-Et-Im][OAc] (**18**), and a modest solubility (2 mg/mL) in [EMIM][$EtSO_4$] (**9**) and [Bu_4N][HCOO] (**2**). On the other hand, very high protein solubilities (> 4–5 mg/mL) were observed in other dca^-, $HCOO^-$ and OAc^- based ILs (especially in **3** and **4**, the free CALB solubility could be as high as 10 mg/mL in some reaction mixtures[60]). The general trend is that higher molar concentrations of anions (such as acetate and formate) lead to higher lipase solubility. Meanwhile, the structure of cations and the overall IL properties also affect the lipase solubility as suggested by Table 2. It is interesting to mention the dissolved lipase in ILs can be precipitated out strongly by the addition of methanol, ethanol or acetone, and weakly by 2-propanol, but not by the addition of DMSO and acetonitrile. This allows the possible use of IL mixtures with organic solvents as media for homogeneous biocatalysis.

The anion molar concentration (M or mol/L) of a pure IL can be estimated from its density (d) and molecular weight (Mw) based on eqn 1 (assuming IL is monoanion),

$$[anion] = [IL] = \frac{m/Mw}{V/1000} = \frac{1000d}{Mw} \quad (1)$$

where m is the mass of IL (g), V is the volume of IL (mL), and d is assumed to be 1.2 mg/mL for all ILs (based on density data of ether-functionalized ILs[29,34]). Our previous study[15] suggested that a higher anion (acetate) molar concentration (*i.e.* lower Mw and a smaller cation of IL) is beneficial to the dissolution of substrates, whereas a lower anion molar concentration (*i.e.* higher Mw and a larger cation) is advantageous for the enzyme stabilization. Therefore, the IL structure can be tailored to exhibit a balanced H-bonding capability: strong enough to dissolve substrates (at high temperatures if necessary) and enzymes, but not too strong to disrupt the protein structures and interact with enzyme's active sites (at moderate or low temperatures). This explanation has been confirmed by the lipase activity data in Table 2. Lower CALB activities were seen in enzyme-denaturing ILs such as [BMIM][dca] (**6**), [EMIM][OAc] (**7**) and [Bu_4N][HCOO] (**2**) due to the strong hydrogen-bonding basicity of anions and high anion concentrations,[15–18,52] which is consistent with previous findings of low enzyme activities in these ILs.[15,17,20] With the increase in cation size, we observed higher lipase activity (both free and immobilized forms) in ILs such as [OMIM][OAc] (**8**), [Amm110]Cl (**10**), [Me(OEt)$_2$-Et_3N][OAc] (**11**), [Me(OEt)$_3$-Et_3N][OAc] (**4**), [Me(OEt)$_3$-Et_3N][HCOO] (**12**), [Me(OEt)$_3$-Et-Im][OAc] (**3**), [Me(OEt)$_3$-Bu-Im][OAc] (**14**), [Me(OEt)$_7$-Et-Im][OAc] (**15**), [Me(OPr)$_3$-Et-Im][OAc] (**17**), and [Me(OEt)$_3$-Me-Et-Im][OAc] (**18**). Free CALB is more active in [Me(OEt)$_3$-Et-Im][dca] (**19**) than the immobilized lipase. Fig 1 illustrates the relationship between the immobilized-CALB activity and the anion molar concentration for a homologous series of acetate ILs (differing in numbers of -OEt- and/or -CH_2- groups). The general trend confirms that a lower anion concentration leads to a higher lipase activity. Of course, as shown in Table 2, the lipase activity bears no simple linear relationship with the anion concentration as the cation and overall IL properties also play critical roles.

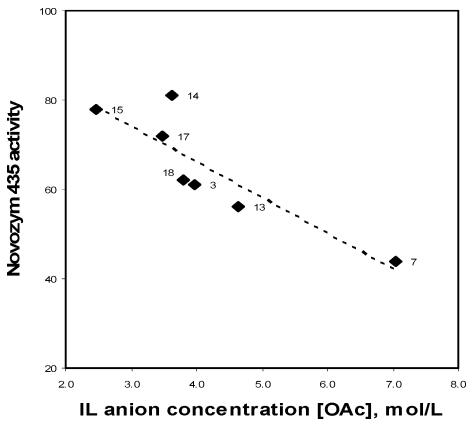


Fig. 1 Effect of anion (acetate) molar concentration on the lipase activity (plot from data of Novozym 435-catalyzed percent yield at 24 h in Table 2; the anion concentrations were estimated from eqn 1; the number near each data point represents the IL number in Table 2).

It is important to address two particular ILs (**18** and **20**) in Table 2. **18** is different from **3** by having an additional methyl group at the C-2 position of imidazolium ring. It is known the C-2 hydrogen of imidazolium is acidic (pK_a = 21–23),[61–63] and replacing this hydrogen by a methyl group is expected to reduce the H-bonding acidity. Based on Table 2, the activities of immobilized CALB in **3** and **18** are comparable, however, free CALB seems more active in **3** than in **18**. The free CALB solubility is reduced from > 5 mg/mL in **3** to 1 mg/mL in **18**. In addition, the viscosity of **18** (239 mPa s at 20 °C, based on method in Ref. 15) is significantly higher than **3** (92 mPa s[15]). The other IL, [EMIM][OTf] (**20**), seems lipase denaturing based on the activity data (Table 2), although several studies achieved the CALB-catalyzed synthesis of sugar esters in [EMIM][OTf] and [BMIM][OTf],[64–66] as well as CALB-catalyzed biodiesel synthesis in [EMIM][OTf].[67] In addition, [EMIM][OTf] (**20**) has a poor dissolution power towards many substances (see Table 3 and discussion in next section).

To address issues of the lipase stability and reusability, immobilized CALB was incubated in **3** at 50 °C for 72 h, and no loss in catalytic capability was observed (Fig 2). In addition, ether-functionalized ILs (**3**, **4**, **11**, **12**, **13** and **19**) have low viscosities (50–150 mPa s at 20 °C), and can dissolve a variety of substrates (see Ref. 15 and Table 3). High CALB activities (both free and immobilized) were also obtained in [EMIM][$EtSO_4$] (**9**), although a low activity was reported previously for the immobilized CALB.[14] The anion ($EtSO_4^-$) has a low

¶ When measuring Circular Dichroism (CD) spectra of enzyme in ILs, the CALB concentration in two Tf_2N^- based ILs was 0.22 mg/mL.[79] The α-chymotrypsin in [EMIM][Tf_2N] was 0.03 mg/mL for fluorescence spectroscopy, and 0.1–0.15 mg/mL for CD spectroscopy.[77]

Table 3 Solubility of different substances in ILs

	IL	Cellulose wt% (110 °C)	D-glucose wt% (60 °C)	DOPA (20 °C)[a]	Betulinic acid (25 °C)
7	[EMIM][OAc]	15%	60% (~4000 mM)	60 mM	40 mM
9	[EMIM][$EtSO_4$]	< 0.5%	30% (~2000 mM)	< 50 mM	< 20 mM
10	[Amm110]Cl	0.5%	20% (~1300 mM)	< 70 mM	< 20 mM
3	[$Me(OEt)_3$-Et-Im][OAc]	12%	80% (~5300 mM)	420 mM	640 mM
4	[$Me(OEt)_3$-Et_3N][OAc]	10%	16% (~1100 mM)	280 mM	300 mM
12	[$Me(OEt)_3$-Et_3N][HCOO]	10%	70% (~4700 mM)	200 mM	50 mM
19	[$Me(OEt)_3$-Et-Im][dca]	< 0.5%	20% (~1300 mM)	< 50 mM	< 20 mM
20	[EMIM][OTf]	< 0.5%	1% (~65 mM)	< 50 mM	< 20 mM

[a] DOPA is 3,4-dihydroxy-DL-phenylalanine.

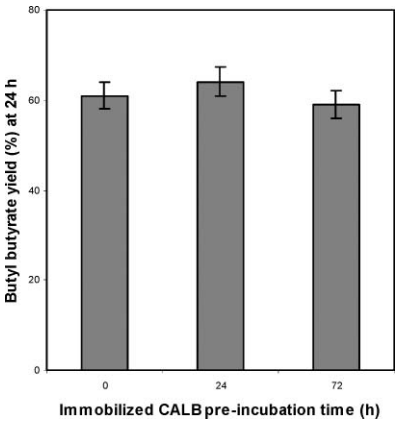


Fig. 2 Stability of immobilized CALB in [$Me(OEt)_3$-Et-Im][OAc] (**3**) at 50 °C (see Experimental section).

hydrogen-bonding strength as implied by the relatively low solubilities of CALB (Table 2) and substrates (Table 3). It is also important to reiterate that there is some leaking of the lipase from Novozym-435 into the ether-functionalized ILs;[15] therefore, the covalent immobilization of CALB deserves a further study in these ILs.

Solubility of different substrates in ether-functionalized ILs

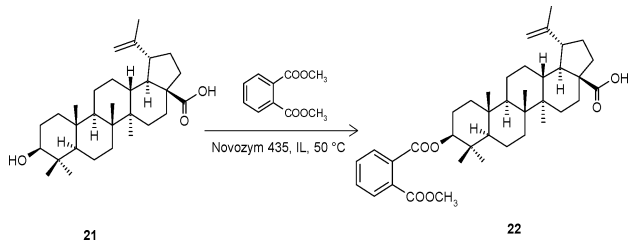
Previously, we observed a low cellulose solubility in ILs **8**, **10**, **14–17** due to their low anion concentrations.[15] In contrast, ILs **3**, **4** and **11–13**, have 'super' dissolution power towards many substances that are difficult to dissolve in conventional organic solvents and/or aqueous solutions (Ref. 15 and Table 3). In this paper, we selectively report the solubilities of several representative substrates in ILs (Table 3). Cellulose is the most abundant natural biomass on earth. However, many cellulose-related processes are hampered by the low solubility of cellulose in most organic solvents. In the past several years, it was discovered that certain ILs can dissolve ~10% (wt) or more cellulose; the main dissolution mechanism is known as the high hydrogen-bonding basicity of anions (such as chloride, formate, acetate or alkylphosphonate).[15,68,69] Experimentally, ^{13}C and $^{35/37}Cl$ NMR relaxation measurements on [BMIM]Cl confirmed that chloride ions interact with the cellulose -OH groups in a 1:1 stoichiometry (-OH $\cdots$ Cl).[70] Molecular dynamics simulation of β-D-glucose in 1,3-dimethylimidazolium chloride ([MMIM]Cl) also reached the same conclusion about the hydrogen bonding between chloride anions and hydroxyl groups (-OH $\cdots$ Cl).[71,72]

As shown in Table 3, [EMIM][$EtSO_4$] (**9**), [Amm110]Cl (**10**), [EMIM][OTf] (**20**), and [$Me(OEt)_3$-Et-Im][dca] (**19**) could barely dissolve any cellulose, mainly because of the poor hydrogen-bonding basicity of $EtSO_4^-$ in **9** and OTf^- in **20**, and a low Cl^- concentration in **10**. It is known dca^- based ILs are poor in dissolving cellulose.[15,33,36] Other acetate and formate-based ILs (**3**, **4**, **7** and **12**) in Table 3 are able to dissolve at least 10% (wt) of cellulose. This makes the enzymatic modification of cellulose in ILs possible.[15]

The solubility of D-glucose in most ILs (Table 3) ranges from 16% (wt) (~1100 mM) to 80% (~5300 mM) at 60 °C. Particularly, the glucose solubility in [$Me(OEt)_3$-Et-Im][OAc] (**3**) is as high as 80% (wt) at 60 °C. Since D-glucose is not quite soluble in most organic solvents, these encouraging outcomes enable the synthesis of glucose derivatives at high concentrations *via* enzymatic routes.[15] In addition, we also observed that IL **3** could dissolve more than 20% (wt) L-ascorbic acid (vitamin C) at 25 °C (data not shown in Table 3). On the other hand, D-glucose has a poor solubility in [EMIM][OTf] (1% or ~65 mM at 60 °), which is consistent with literature values (30.6 mM in [BMIM][OTf] at 40 °C,[65] 6.1 g/L or 34 mM in [EMIM][OTf] and 4.8 g/L or 27 mM in [BMIM][OTf] at 25 °C;[64] supersaturated concentrations in OTf^- ILs range from 113–363 mM[64,65]). The dca^- based IL (**19**) dissolved a high concentration (20%) of D-glucose, but could dissolve little other compounds (see Table 3).

Amino acids, as important Active Pharmaceutical Ingredients (APIs), are another type of compounds that are usually soluble in water but not soluble in most organic solvents without proper *N*-protection. Therefore, a direct (chemical or enzymatic) modification of amino acids in organic solvents is not efficient. We selected an important amino acid known as DOPA (3,4-dihydroxy-DL-phenylalanine) to demonstrate its solubility in ILs (more comprehensive study of other amino acids will be reported elsewhere). Its enantiomer, L-DOPA is able to increase dopamine levels for the treatment of Parkinson's disease and DOPA-Responsive Dystonia. As shown in Table 3, DOPA has a poor to moderate solubility in **7**, **9**, **10**, **19** and **20**, but has a very high solubility in **3**, **4** and **12** (200–420 mM).

Betulinic acid (3β-hydroxy-lup-20(29)-en-28-oic acid) (**21**) (Scheme 3) is a pentacyclic lupane-type triterpene found in the bark of some trees (such as birch). This compound and its derivatives have exhibited a variety of biological properties such as anticancer, anti-HIV, antibacterial, anti-malarial, anti-inflammatory, anthelmintic activities.[73,74] However, betulinic acid has very low solubilities in water and common organic solvents (such as alcohols, ethers and esters), which creates a



Scheme 3 Synthesis of methyl-phthalate of betulinic acid (**22**) *via* enzymatic transesterification in [Me(OEt)$_3$-Et-Im][OAc] (**3**).

huge barrier for a further chemical or enzymatic modification of this compound. For example, its solubility in water is only about 0.02 μg/mL at room temperature.[75] We also measured the solubility in some organic solvents at 25 °C: 1% (wt/v) in ethanol, 5% (wt/v) in DMSO, and > 23% (wt/v) in pyridine. The high solubility in pyridine is due to the high basicity of the solvent; however, many chemical and enzymatic reactions can not be carried out in pyridine. On the other hand, betulinic acid is very soluble in ILs **3** and **4** (640 and 300 mM respectively at 25 °C, Table 3). This creates a new opportunity for derivatizing this naturally occurring compound in ionic media at high concentrations. The enzymatic conversions of some of these molecules in ether-functionalized ILs are illustrated in a later section.

FT-IR and fluorescence determination of protein structures in ILs

The infrared (IR) spectra of proteins can reveal characteristic amide bands from different vibrations of the peptide moiety. Among different amide modes of the peptide group, the most common mode is the amide I, which is the C=O stretching vibration of the amide group between the region of 1600 and 1700 cm^{-1}. The second derivatives of IR spectra in this region are often used to study the protein's secondary structures in terms of structural elements such as α-helix, β-sheets, β-turns, and non-ordered or irregular structures.[76] The α-helical structures are normally shown between 1650 and 1658 cm^{-1}, and β-sheets are between 1620 and 1640 cm^{-1}.[76] The assignments of infrared amide I band components with various secondary protein structures are summarized from the literature (Table S.1 in ESI†).

As shown in Fig 3(a) and (b), after incubated in ILs **3** and **4** respectively for 24 h at 50 °C, free CALB has essentially the same second derivative spectra as that in water, suggesting these two ILs do not disrupt the protein's secondary structures. A similar conclusion was observed in two other ILs [OMIM][OAc] (**8**) and [EMIM][EtSO$_4$] (**9**) (see ESI,† Fig. S.1). However, second derivative spectra of free CALB incubated in [EMIM][OAc] (**7**), [BMIM][HCOO] (**2a**) and [Me(OEt)$_3$-Et$_3$N][HCOO] (**12**) suggest moderate to severe structural changes: band shifting and uncharacteristic structures at 1623 (β-sheets) and 1678 cm^{-1} (β-turns) in IL **7** (Fig 3c), loosing characteristics at 1623 (β-sheets), 1664 (β-turns) and 1671 cm^{-1} (β-turns) in IL **2a** (Fig 3d), and disappearing α-helix and β-sheets structures in IL **12** (Fig S.1). These structural changes are generally in agreement with the lipase activities in ILs (Table 2): high enzyme activity in ILs **3**, **4**, **8** and **9**, but low activity in **2a** and **7**. It also confirms our previous finding that lower anion molar concentration of IL is more enzyme-stabilizing (such as **3** and **8** are more enzyme-compatible than

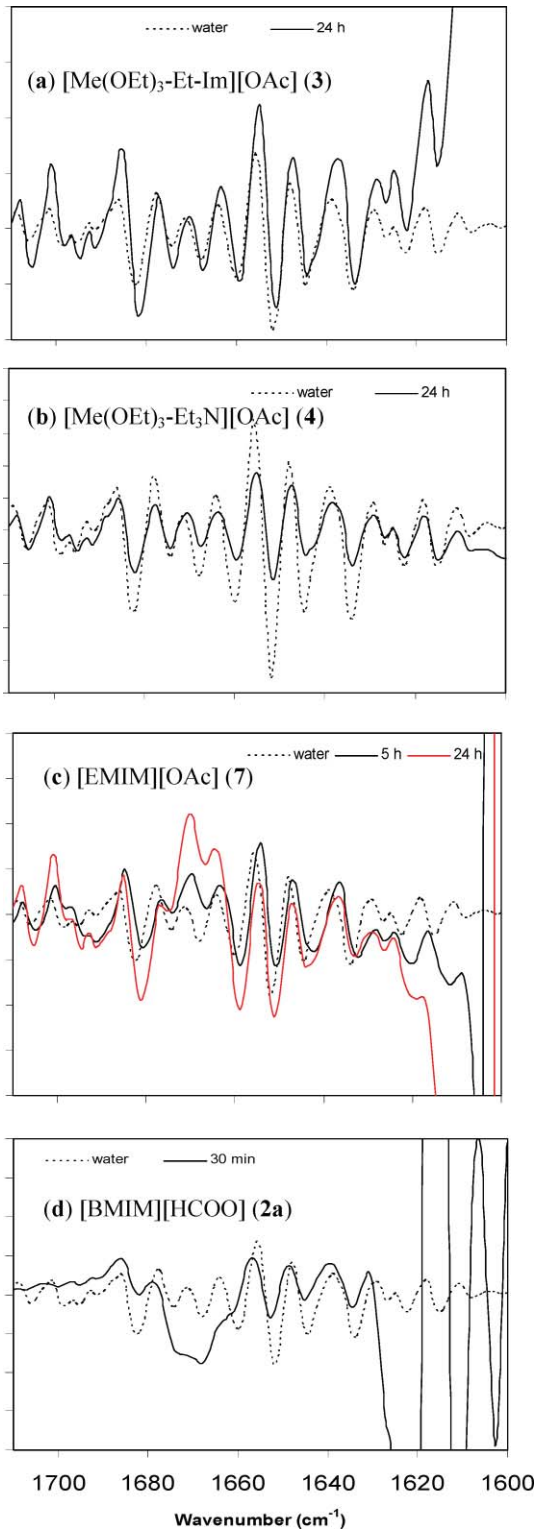


Fig. 3 Second derivative ($d^2A/d\nu^2$) spectra of free CALB in water (dashed line) and in ILs (solid lines, with different incubation times at 50 °C).

7 although they all contain acetate anions; **4** is more enzyme compatible than **11**). Based on the infrared spectrum (Fig S.1c), IL **12** is a protein structure breaker and enzyme denaturant. The free lipase in this IL is only moderately active, but it seems that the immobilized enzyme could tolerate this IL (Table 2).

In summary, the presence of low molar concentrations of denaturing anions (such as acetate) does not cause much change of protein's secondary structures. The ether-functionalized ILs **3** and **4** are lipase-compatible based on both activity data and IR secondary derivatives.

On the other hand, it is also important to realize that the enzyme may not be catalytically active even if its secondary structures are well preserved in the solvent. [BMIM][dca] (**6**) is known an enzyme-denaturing IL (Table 2 and Refs. 15,17), however, the second derivative spectrum of CALB incubated in **6** for 4 days is very similar to that of native lipase (Fig S.1d).

Fluorescence and CD spectroscopic methods have been successfully applied in analyzing the structures of α-chymotrypsin[77,78] and CALB[78,79] in hydrophobic ILs including [EMIM][Tf_2N] and [$BuMe_3N$][Tf_2N]. However, a recent study[80] suggests that fluorescence and CD spectroscopy is incapable of determining the protease's structures in hydrophilic [MMIM][Me_2PO_4] (1,3-dimethylimidazolium dimethylphosphate) due to the interference of IL. Our fluorescence data (Fig S.2 in ESI†) also confirm that the emission maximum (325 nm) of tryptophan (Trp) residues observed in water could not be detected in ILs (**3**, **4** and **9**) even after pure IL signals were subtracted. CALB is a known monomeric protein (33 kDa) containing five Trp, nine Tyr, and ten Phe residues and four disulfide bridges; three Trp residues (positions 52, 113, and 155) are found in three different α-helices, while two other residues (positions 65 and 104) are located in two different β-sheets.[81,82] In our fluorescence measurements, it is suspected that IL molecules interact with Trp residues, causing the Trp emission maximum not shown. However, a further study is needed to understand the mechanisms of such interactions.

Enzymatic transesterification of betulinic acid

Due to the low solubility of betulinic acid in many organic solvents, its synthesis and derivatization are limited to several solvents, such as dichloromethane, chloroform, acetone, THF, DMF, pyridine, benzene, *etc.*[83–85] Most of these solvents (except pyridine) do not dissolve significant amounts of betulinic acid. Therefore, the efficiency of many reactions is limited by the poor reactant solubility. However, as discussed previously, ILs (such as **3** and **4**) can dissolve high concentrations of betulinic acid even at ambient temperature. Unlike pyridine being basic, these ILs are neutral and even compatible with the lipase. For these reasons, ether-functionalized ILs (**3** and **4**) could be suitable media for chemical and enzymatic modification of betulinic acid.

To demonstrate this concept, we conducted the CALB-catalyzed synthesis of methyl-phthalate of betulinic acid (**22**) in IL **3** *via* the transesterification of betulinic acid (**21**) and dimethyl phthalate (Scheme 3). Hemiphthalic esters have higher cytotoxicity than betulinic acid, but the chemical synthesis of **22** was reported as a multi-step strategy.[85] In our enzymatic route, a high substrate concentration (110 mM) was used in the one-step reaction, and a high isolated yield (85%) was achieved (see Experimental for procedures and product characterization). The chemical derivatization of betulinic acid in ILs is being pursued in our laboratory.

Lipase-catalyzed synthesis of fatty acid ester of D-glucose

Fatty acid sugar esters, as biodegradable nonionic surfactants, have many applications in cosmetic, pharmaceutical, and food industry. However, the enzymatic synthesis of these esters is challenged by the selection of an appropriate solvent: most organic solvents are not able to dissolve the highly polar sugar and the nonpolar fatty acid at the same time. A few solvents (such as pyridine) may serve this purpose, but they often cause enzyme denaturation. Common organic solvents for enzymatic transformations of D-glucose include *t*-butanol[45] and 2-methyl-2-butanol.[86] However, sugar solubilities in these solvents are usually low (such as < 20 mM D-glucose in 2-methyl-2-butanol at 60 °C) although the supersaturated sugar solution could be prepared (50 mM D-glucose in 2-methyl-2-butanol at 60 °C).[86] The supercritical CO_2 was explored as the alternative solvent for the CALB-catalyzed synthesis of fructose palmitate, where improved lipase thermal stability and conversions were observed.[87,88] However, the solubility of sugars in nonpolar CO_2 is usually low; therefore, a polar co-solvent (such as *t*-butanol) is usually added.[87] Recently, it was observed that ILs based on BF_4^-, OTf^-, dca^- and OAc^- could dissolve high concentrations of sugars.[15,33,89] Among these ILs, BF_4^- based ILs (or mixtures with OTf^-, or Tf_2N^-) are often used in dissolving sugars for enzymatic synthesis[25,64,65,90] because BF_4^- anions in low-water systems are less enzyme-denaturing than other anions.[20] However, the sugar solubility in BF_4^- based ILs is moderately low.

Since our lipase-compatible ILs (**3** and **4**) can dissolve high concentrations of D-glucose (Table 3), we carried out the synthesis of fatty acid esters in these ILs or their mixtures with *t*-butanol. High concentrations of D-glucose (220 mM) and vinyl laurate (330 mM) are fully soluble in ILs (**3** and **4**) or their mixtures with *t*-BuOH, demonstrating the advantage of employing these ionic solvents. In general, with the increase of *t*-BuOH concentration (trials 1, 2 and 4 in Table 4), the D-glucose conversion decreased, suggesting IL **3** is more enzyme stabilizing than *t*-BuOH. Even in 40% IL **3** and 60% *t*-BuOH (trial 4), D-glucose (220 mM) and vinyl laurate (330 mM) are fully soluble; therefore, the limitation of substrate solubility is unlikely the reason of lower conversion in high concentrations of *t*-BuOH. The use of molecular sieves to scavenge water is a common strategy to increase the acylation yield.[25,64] However, the addition of molecular sieves into our ionic solvent (containing 60% IL **3** and 40% *t*-BuOH, trial 3) only resulted in a marginal increase in D-glucose conversion (from 79% to 82%). We rationalized two possible reasons: (1) Our solvents and

Table 4 Enzymatic transesterification of D-glucose and vinyl laurate

Trial	Solvent (IL/*t*-BuOH volume ratio)	D-glucose conversion at 24 h
1	IL **3**/*t*-BuOH 100 : 0	85%
2	IL **3**/*t*-BuOH 60 : 40	79%
3	IL **3**/*t*-BuOH 60 : 40 (molecular sieves, 0.2 g)	82%
4	IL **3**/*t*-BuOH 40 : 60	68%
5	IL **4**/*t*-BuOH 100 : 0	71%

Reaction conditions: 1.0 mL solvent (IL used was **3** or **4**), 0.22 mmol D-glucose, 0.33 mmol vinyl laurate, 40 mg Novozym 435 and 50 °C. Molecular sieves (3A) were not added except in trial 3.

Table 5 Anomerization ratios of D-glucose in different solvents as determined by ^{13}C NMR

Solvent	β-D-glucose (%)	α-D-glucose (%)
2-methyl 2-butanol[86]	40	60
D_2O	64	36
IL 3	76	24
IL 3 (52 days at r.t.)	71	29
IL 4	83	17
IL 4 (52 days at r.t.)	85	15

substrates were dried over $MgSO_4$ (for *t*-BuOH and substrates) or molecular sieves (for ILs), and immobilized CALB was also intensively dried over P_2O_5; therefore, the initial water content was extremely low. (2) Our hydrophilic ILs absorbed water produced by the reaction; consequently these water molecules are not 'freely' available for hydrolyzing D-glucose fatty acid ester. The same reaction in IL **4** (trial 5) showed a lower glucose conversion than that in IL **3** (trial 1). Our recent study[15] has confirmed that the CALB-catalyzed acylation of D-glucose in these ILs is highly regioselective and occurs at the C-6 position of D-glucose.

However, the acylation reactions in ILs **3** and **4** were relatively slow and 24 h is usually needed even with vinyl laurate as the acyl donor. On the other hand, the same reaction was observed faster in organic solvents (such as 2-methy-2-butanol[86]) and in other ILs (such as [BMIM][OTf][64] or its mixture with [BMIM][Tf_2N][65]), where most of the product was obtained in 10 h (although these solvents could dissolve much less glucose than **3** and **4**). Previously, we also reported low enzymatic conversions of D-glucose when reacting with methyl methacrylate in **3** and **4**.[15] We suspected that since D-glucose is very soluble in these ionic media, the substrate ground-state stabilization might be the reason behind the slower reaction rates. To articulate this speculation, we dissolved ^{13}C-labeled D-glucose ($^{13}C_6$) in D_2O, IL **3** and **4** respectively, and then determined the anomer ratios of D-glucose (see Figs. S.3 and S.4 in ESI,† and Table 5) in these solutions from ^{13}C spectrum integrations (based on C_1). When dissolving in 2-methyl-2-butanol, D-glucose has 40% β-anomer and 60% α-anomer.[86] In water (D_2O), our NMR measurements suggest there are more β-anomer (64%) than α-anomer (36%), which is consistent with the known literature values.[91] In two ionic solutions (**3** and **4**), there are about 80% β-D-glucose anomers and only 20% α-anomer (after 52 days of incubation at room temperature, there are only slight changes of the ratios; see Table 5). It is considered that strong solvation effects account for the high abundance of β-anomers in water,[92] and β-glucose has a lower free energy and thus more stable than α-anomer.[91] Based on this theory, D-glucose is highly solvated in ILs **3** and **4**, presumably due to the hydrogen bond network between IL molecules and D-glucose. We also suspected that during the enzymatic (trans)esterification, α-anomer is more reactive due to the higher substrate ground-state energy. This speculation is supported by the CALB-catalyzed esterification of D-glucose with dodecanoic acid in *t*-butanol, where much more α-anomer ester was produced than the β-form (α/β = 84/14 from GC analysis).[45] Although Flores *et al.*[86] observed both anomeric forms of glucose were consumed at the same rate in the enzymatic acylation with dodecanoic acid, they rationalized that it was due to the rate of mutarotation is faster than the reaction rate in 2-methyl-2-butanol. However, in ionic solutions (**3** and **4**), the interconversion of two anomeric forms of D-glucose is expected slow because D-glucose molecules form strong hydrogen bonds with anions.[69–72] The NMR spectrum of our product shows the chemical shift of glucose H1 is about 5.50 ppm (see ESI†), suggesting the ester is α-D-glucose laurate.[45]

Recent reports[93,94] suggest that the imidazolium-based ILs (such as [EMIM][OAc] and [BMIM][OAc]) may react with glucose (and reducing end group of cellulose) at C-2 position of the imidazolium ring. After incubation in imidazolium IL **3** for 52 days at room temperature, D-glucose-$^{13}C_6$ showed two tiny new ^{13}C peaks at 63.7 and 64.3 ppm (see ESI,† Fig S.3c), suggesting the side reaction between D-glucose and IL **3** occurred but insignificant. IL **3** has a long and flexible alkyloxyalkyl chain than [EMIM][OAc] and [BMIM][OAc], which could create steric hindrance for D-glucose to react with **3**. On the other hand, after dissolving in ammonium-type IL **4** for 52 days at room temperature (see ESI,† Fig S.4c), D-glucose showed no new ^{13}C peaks, implying no reaction between these two substances.

Conclusions

Ether-functionalized ILs were found capable of dissolving free CALB, and stabilizing the enzyme at the same time. The infrared spectra of lipase confirmed that these ILs do not disrupt the secondary structures of proteins. These new ionic solvents have 'super' dissolution ability towards a variety of substrates including sugars, cellulose, L-ascorbic acid, betulinic acid and amino acids. To demonstrate the potential biotransformation of these substrates in ionic media, we carried out two different lipase-catalyzed reactions to synthesize methyl-phthalate of betulinic acid and D-glucose fatty acid esters, respectively. The success of these reactions can be attributed to the high substrate solubilities in ILs and the enzyme-stabilizing nature of ionic media. However, we should cautiously examine if the high substrate solubility in ILs may also cause the ground-state stabilization of reactants (resulting in less-reactive substrates), and if there are side reactions between ILs and substrates (such as the case of imidazolium ILs reacting with D-glucose and cellulose).

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