"Water-like" Ammonium-Based Ionic Liquids for Lipase Activation and Enzymatic Polymerization

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Abstract

Novel dual-functionalized ammonium-based hydrophobic ionic liquids (ILs) have been synthesized by mimicking the water structure to carry both ether group (hydrogen-bond acceptor) and *tert*-alcohol group (hydrogen-bond donor). These ammonium-type ionic solvents exhibit the advantage of lower viscosities (as low as 129 mPa s at 30 °C) than the imidazolium analogue (~300 mPa s at 30 °C); more importantly, these "water-like" media are highly compatible with immobilized *Candida antarctica* lipase B (Novozym 435) in terms of producing high transesterification activities (1.5-fold higher than that in *tert*-butanol, and slightly higher than that in diisopropyl ether) and higher thermal stability than that in *tert*-butanol. To demonstrate the utilization of this new enzymatic system, enzymatic ring-opening polymerization (ROP) of ε-caprolactone using these "water-like" ILs as co-solvents was carried out to synthesize polyesters, achieving high molecular mass M_w (up to 18,000 Da) and high yields (up to 74%).

Keywords: ionic liquid; biocatalysis; enzyme activation; enzymatic ring-opening polymerization

1. Introduction

Nonaqueous biocatalysis in organic solvents has drawn a great deal of attention since some pioneering work in 1980s [1-8]. Obvious advantages of enzymatic reactions in nonaqueous media include high enantioselectivity, high thermal stability, substrate dissolution in organic solvents, reversing hydrolysis reactions to become synthesis, and simple recovery of enzyme and product, etc. [9-13]. However, nonaqueous biocatalysis has been challenged by large-scale applications for issues like high enzyme cost, protein fragility, and depressed enzyme activity. In particular, enzyme activity in nonaqueous media is significantly lower than that in aqueous solutions [9, 14]. For instance, α-chymotrypsin and subtilisin in octane were 10^4–10^5 times less active than in water [9]. Likely explanations for activity depression include the limitation of substrate mass transfer to insoluble enzymes in organic media, poor accessibility to active sites of lyophilized or cross-linked enzyme particles, structural changes of enzyme molecules, unfavorable energetics of substrate desolvation (i.e. enzyme-substrate binding is weaken due to the tendency of substrate staying in organic phase) and transition state stabilization (i.e. water stabilizes highly polar transition state much better than organic solvents), reduced conformational mobility, and poor pH optimization [14]. Therefore, a careful design of water-mimicking nonaqueous solvents could lead to transition state stabilization, higher conformational mobility of enzymes, and improved enzyme-substrate binding.

Ionic liquids (ILs) represent a new group of highly designable nonaqueous solvents that could be compatible with enzymes [15-19]. A number of groups have indicated that enzymes displayed high activities and/or enantioselectivity in hydroxyl- or ether-functionalized ILs; these functionalized ionic solvents include various alkoxy-containing Ammoeng type ILs (e.g. cocosalkyl pentaethoxy methylammonium methylsulfate) [20-28], tetrakis(2-

hydroxyethyl)ammonium triflouromethanesulfonate [29], [Me(OCH_2CH_2)$_n$-Et-IM][OAc] and [Me(OCH_2CH_2)$_n$-Et_3N][OAc] [30-32], [$MeOCH_2CH_2$-Bu_3P][Tf_2N] [33-35], [$HOCH_2CH_2$-MIM][PF_6] and [Me(OCH_2CH_2)$_2$-MIM][PF_6] [36], and [Me(OCH_2CH_2)$_n$-Et-IM][Tf_2N] and [Me(OCH_2CH_2)$_n$-Et_3N][Tf_2N] [37, 38], etc. Based on this perspective, our group [39] recently constructed dual-functionalized imidazolium-based ILs containing both groups of *tert*-alcohol and ether to mimic the water structure carrying both hydrogen-bond donating (–OH) and accepting (R–O–R) properties. *tert*-Alcohol groups are selected instead of 1° and 2° alcohols due to the fact that 3° alcohols are more enzyme-compatible (less enzyme inhibition) and much less reactive as substrates in enzymatic reactions especially under dried conditions [1, 40]. In these dual-functionalized imidazoliums, we evaluated the transesterification activity of immobilized lipase B from *Candida antarctica* (CALB), and observed high synthetic activity of CALB: up to 2–4 folds higher than those in non-functionalized 'classic' ILs (e.g. [BMIM][Tf_2N]), and up to 40–100% higher than those in diisopropyl ether and *tert*-butanol. One area needing improvement is that these imidazolium-ILs have relatively high dynamic viscosities (in the neighborhood of 300 mPa s at 30 °C) [39]. The present study aims to prepare novel 'water-like' dual-functionalized ILs containing a different cation core (i.e. alkylammonium) to achieve high lipase activities and lower viscosities at the same time.

2. Materials and methods

2.1. Materials

(2-Methoxyethyl)methylamine, bis(2-methoxyethyl)amine, and 1,1-dimethyloxirane (known as isobutylene oxide; sometimes referred as 2,2-dimethyloxirane) were acquired from TCI America (Portland, OR). Lithium bis(trifluoromethylsulfonyl)imide (Li[Tf_2N]) was the product of Matrix Scientific (Columbia, SC). 1-Butyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide

([BMIM][Tf_2N], synthesis grade) was produced by Merck KGaA (EMD Millipore Corporation, Billerica, MA). Novozym 435 (*Candida antarctica* lipase B (CALB) immobilized on acrylic resin) (Catalog # L4777, Lot # SLBW1544, enzyme activity 11,900 propyl laurate units (PLU)/g) was purchased from Sigma-Aldrich (St. Louis, MO). The synthesis and characterizations of *tert*-alcohol- and ether-functionalized ammonium-based ILs (see Schemes 1 and 2) were described in *Supporting Information*.

2.2. Enzymatic transesterification of ethyl sorbate with 1-propanol

In a capped 3-mL glass vial reactor, 50 μL stock solution of 100 mM ethyl sorbate in 1-propanol was mixed with 1.0 mL of IL. Ethyl sorbate and 1-propanol had final concentrations of 5 mM and 0.67 M, respectively. Following the addition of 20 mg Novozym 435 (assuming containing ~4 mg free CALB for calculation of reaction rates; according to literatures [41-44], free CALB loading in Novozym 435 ranges from 8.5 to 20 wt%), the reaction mixture was incubated in an oil bath at 50 °C and gently stirred. Periodically (each 15 min of the first hour), an aliquot (50 μL) was carefully withdrawn from reaction mixture (while minimizing the removal of enzyme beads) and mixed with 1.0 mL methanol. The diluted sample was centrifuged for about 2 min, and the clear supernatant was added into an autosampler vial for HPLC analysis. The content of propyl sorbate was estimated from its integrated peak area by using the standard curve for ethyl sorbate (propyl sorbate is not commercially available). Since our earlier study [31] suggested that trace amounts of sorbic acid and sorbate ester could migrate out of Novozym 435 beads into various solvents including ILs, control experiments were performed in the absence of substrate (ethyl sorbate) but with the addition of 50 μL 1-propanol. All lipase activities reported herein were net activities after subtracting control rates. Reaction samples were analyzed by a Shimadzu LC-20AD HPLC with an auto-sampler and a SPD-20A UV–Visible dual-wavelength detector at 258

nm. The stationary phase was a Phenomenex® Kinetex C18 column (100 mm × 4.6 mm, particle size 2.6 mm), and the mobile phase was an isocratic eluent comprising methanol and water (60/40, v/v) at 1.0 mL min^{-1} flow rate.

2.3. Enzymatic ring-opening polymerization (ROP) of ε-caprolactone

In a glass reaction vial, ε-caprolactone (0.5 mL with density of 1.03 g/mL) was mixed with 0.25 mL of IL and 100 mg of Novozym 435. The capped vial was incubated in a 70 ºC-oil bath and stirred at 210 rpm. After 48 h, the reaction mixture was cooled to room temperature, followed by the addition of 2.0 mL of chloroform to solubilize the polyester with a small spatula breaking apart the solid. The enzyme beads were removed by vacuum filtration with additional chloroform to wash the solid. Chloroform in filtrate was evaporated and then methanol was added to precipitate the polymer, which was collected by centrifugation or vacuum filtration. The white polyester product was air-dried for 24 h.

Polyester yield was calculated by dividing polyester mass with ε-caprolactone mass. Mass-average molecular mass (M_w) and polydispersity index (PDI = M_w/M_n) of polymers were obtained from analyses by a GPC (LC-20AD Shimadzu HPLC) with a SPD-20A UV–visible dual-wavelength detector at 210 and 254 nm, and two Agilent PLgel MIXED-B (10 μm, 300 × 7.5 mm) columns eluted with 1.0 mL/min THF at 30 ºC [45]. The calibration curve was generated by polystyrene standards with M_w in the range of 1,130 to 62,500 Da [46].

3. Results and discussion

To synthesize dual-functionalized ammonium-based ILs, our initial attempts (as illustrated by Scheme S1(a) in *Supporting Information*) began by grafting an ethyl group or *tert*-alcohol group onto diethylamine to yield tertiary amines. However, further quaternarization reactions to append an additional functional group failed, most likely due to steric hindrance that is unfavorable for

nucleophilic alkylation; similar failures were found for two other approaches [Schemes S1(b) and (c)]. To move forward, we modified our synthetic strategy by starting with an ether-functionalized secondary amine (such as (2-methoxyethyl)methylamine, or bis(2-methoxyethyl)amine). These ether-functionalized amines were purchased from a commercial source, but could be prepared by reacting glycol with *p*-toluenesulfonyl chloride to form sulfonate ester which further reacts with a primary or methyl amine [47]. As shown in Scheme 1, ether-functionalized amine reacted with 1,1-dimethyloxirane to yield a tertiary amine grafted with a *tert*-butyl alcohol group, which could be easily converted to quaternary ammonium salt by refluxing with iodomethane in acetone (however, refluxing with bromoethane took 48 h at a higher temperature in acetonitrile). The resulted ammonium halide was converted to Tf_2N^- hydrophobic IL through an anion exchange (Scheme 1), which was purified by rinsing with diethyl ether and/or *n*-heptane to remove impurities. Three ILs (Scheme 2) produced are [$CH_3OCH_2CH_2$-Me_2N-*t*-BuOH][Tf_2N] (**1**), [$(CH_3OCH_2CH_2)_2$-MeN-*t*-BuOH][Tf_2N] (**2**), and [$CH_3OCH_2CH_2$-Me-EtN-*t*-BuOH][Tf_2N] (**3**).

The synthetic activity of Novozym 435 (immobilized *Candida antarctica* lipase B (CALB)) in different solvents was evaluated by a highly sensitive transesterification reaction between ethyl sorbate and 1-propanol [31, 35, 38, 39]. Lipases are known to have high synthetic activities in *tert*-butanol [40, 48-53] and diisopropyl ether [54-57], and thus these two organic solvents are often used as 'baselines' for comparing enzyme's synthetic activities. As illustrated in Table 1, Novozym 435 displayed high activities of 5.94 and 8.57 μmol min^{-1} g^{-1} CALB in *tert*-butanol (trial 1) and diisopropyl ether (trial 2) respectively. In the absence of Novozym 435, no appreciable transesterification could be detected in organic solvents or any ILs listed in Table 1. A non-functionalized 'classical' hydrophobic IL ([BMIM][Tf_2N], trial 3) is well-known for its

high compatibility with enzymes [15, 18, 58], which afforded the transesterification activity of 5.12 μmol min^{-1} g^{-1} CALB in the present study. A dual-functionalized imidazolium IL ([$CH_3OCH_2CH_2$-Im-*t*-BuOH][Tf_2N], trial 4, recently synthesized by our group) exhibited a very high enzyme activity of 12.36 μmol min^{-1} g^{-1} CALB [39]. Trials 5a-d demonstrate the effect of water contents on CALB activities in [$CH_3OCH_2CH_2$-Me_2N-*t*-BuOH][Tf_2N] (**1**): lipase activity increased from 0.005 wt% to 0.02 wt% H_2O, and then declined at 0.03 wt% H_2O. These results imply the necessity of a small amount of water (sometime knowns as 'essential water' [3, 59]) in nonaqueous media to activate the enzyme. The highest CALB activity achieved in this IL (**1**) was 9.15 μmol min^{-1} g^{-1} CALB (with 0.02 wt% H_2O). A similar trend was observed in trials 6a-b and 7a-b where lipase activities were lower when the water content was below 0.02 wt%. Highest activities observed for [($CH_3OCH_2CH_2$)$_2$-MeN-*t*-BuOH][Tf_2N] (**2**) and [$CH_3OCH_2CH_2$-Me-EtN-*t*-BuOH][Tf_2N] (**3**) were 6.73 and 7.37 μmol min^{-1} g^{-1} CALB (with 0.02 wt% H_2O) respectively. New dual-functionalized ammonium-based ILs (**1**-**3**) enabled excellent lipase activities that are higher than the performance in *tert*-butanol (up to 1.5 fold) and are slightly higher than that in diisopropyl ether. Although these activities (6.73–9.15 μmol min^{-1} g^{-1} CALB in trials 5c, 6b and 7b) are not as high as that in the imidazolium analogue (trial 4), these ammonium-type ILs exhibit the advantage of significantly lower viscosities (129.3–190.4 mPa s at 30 °C) than its imidazolium cousin (303.0 mPa s).

When compared with water and conventional organic solvents, ILs are viscous fluids (mostly in the range of 30–1000 mPa s at room temperature) [60]. Typically, enzymes are suspended in organic solvents or neat ILs during biocatalytic processes, resulting in heterogeneous reaction systems. For this reason, internal and external mass-transfer limitations could become a bottleneck of fast enzymatic reactions [61]. For example, Lozano et al [62] noted

that other than IL polarity, IL viscosity could influence α-chymotrypsin's activity; they observed a higher transesterification activity in less viscous [EMIM][Tf_2N] (34 mPa s) than in much more viscous [MTOA][Tf_2N] (574 mPa s) (MTOA = methyl trioctylammonium). van Rantwijk and Sheldon [18] explained that conformation changes of proteins are slower in a more viscous medium, enabling enzymes to conserve their native structures and activity. In addition, from the industrial processing point of view, a lower solvent viscosity usually leads to an improved operability especially for biocatalytic reactor design [63].

Furthermore, Novozym 435 showed greater thermal stability in ILs (**1**-**3**) than in *tert*-butanol (Fig. 1). At 50 °C, CALB only kept 17% of its synthetic activity in *tert*-butanol after 24 h incubation. However, the lipase retained about 50% activity after 24 h in [$CH_3OCH_2CH_2$-Me_2N-*t*-BuOH][Tf_2N] (**1**), 35% activity in [$(CH_3OCH_2CH_2)_2$-MeN-*t*-BuOH][Tf_2N] (**2**), and 47% activity in [$CH_3OCH_2CH_2$-Me-EtN-*t*-BuOH][Tf_2N] (**3**). Among these three ILs, [$CH_3OCH_2CH_2$-Me_2N-*t*-BuOH][Tf_2N] (**1**) appears to be most compatible with the lipase especially after CALB incubation for 48 h at 50 °C, where 52% residual enzyme activity was detected. This thermal stability of CALB is slightly inferior to that in the imidazolium analogue (i.e. [$CH_3OCH_2CH_2$-Im-*t*-BuOH][Tf_2N]), where 81% and 69% residual activities were observed respectively after 24 h and 48 h incubation at 50 °C [39]. Our earlier study [35] indicated that when Novozym 435 was incubated in [$CH_3OCH_2CH_2NEt_3$][Tf_2N] for 24 or 48 h, its thermal stability at 70 °C was about the half of that at 50 °C; for 24 h-incubation in *tert*-butanol, lipase residual activities were 17% and 1% respectively at 50 °C and 70 °C. Thus, earlier and current results suggest that the lipase CALB exhibited much higher thermal stability in functionalized ILs than in *tert*-butanol.

Additionally, we evaluated these dual-functionalized ILs as co-solvents for enzymatic ring-opening polymerization (ROP) of ε-caprolactone (Scheme 3 and Table 2). Enzymatic ROP

is becoming an attractive and benign route for polyester synthesis [34, 35, 64]. Under solvent-free condition (trial 1 in Table 2), moderate molecular mass (M_w) of 13,800 Da and isolated yield of 37% were obtained. An ether-functionalized imidazolium [$CH_3OCH_2CH_2$-Im-Et][Tf_2N] (trial 2) showed a minimum impact on the polymerization while an ether-functionalized ammonium (trial 3) was able to increase M_w to 17,300 Da but decrease the yield to 11%. On the other hand, dual-functionalized imidazolium and ammonium-based ILs (trial 4-7 in Table 2) considerably improved ROP yields (64–76%) and increased M_w at various degrees (15,800–18,000 Da). The top-performing IL identified was [$CH_3OCH_2CH_2$-Me_2N-*t*-BuOH][Tf_2N] (**1**), achieving M_w = 18,000 Da and 74% isolated yield. Polydispersity index (PDI = M_w/M_n) was generally reduced with the use of ILs (PDI = 1.39–1.66) when comparing with solvent-free condition (PDI = 1.71), implying that ionic co-solvents promoted more uniform enzymatic polymerization.

4. Conclusions

We have synthesized three dual-functionalized ammonium-based ILs carrying both ether and *tert*-alcohol groups. These novel ionic solvents have lower viscosities than the imidazolium analogue. These ammonium-based ILs are highly compatible with Novozym 435 leading to higher transesterification activities than those in [BMIM][Tf_2N] and organic solvents (e.g. *tert*-butanol and diisopropyl ether), and higher thermal stability of CALB than that in *tert*-butanol. When these ILs were employed as co-solvents for enzymatic ROP of ε-caprolactone, high M_w (up to 18,000 Da) and high yields (up to 74%) were obtained. In summary, we have prepared "water-like" ionic solvents that not only mimic the structure of water, but also provide a benign environment for achieving high enzyme activity and stability.

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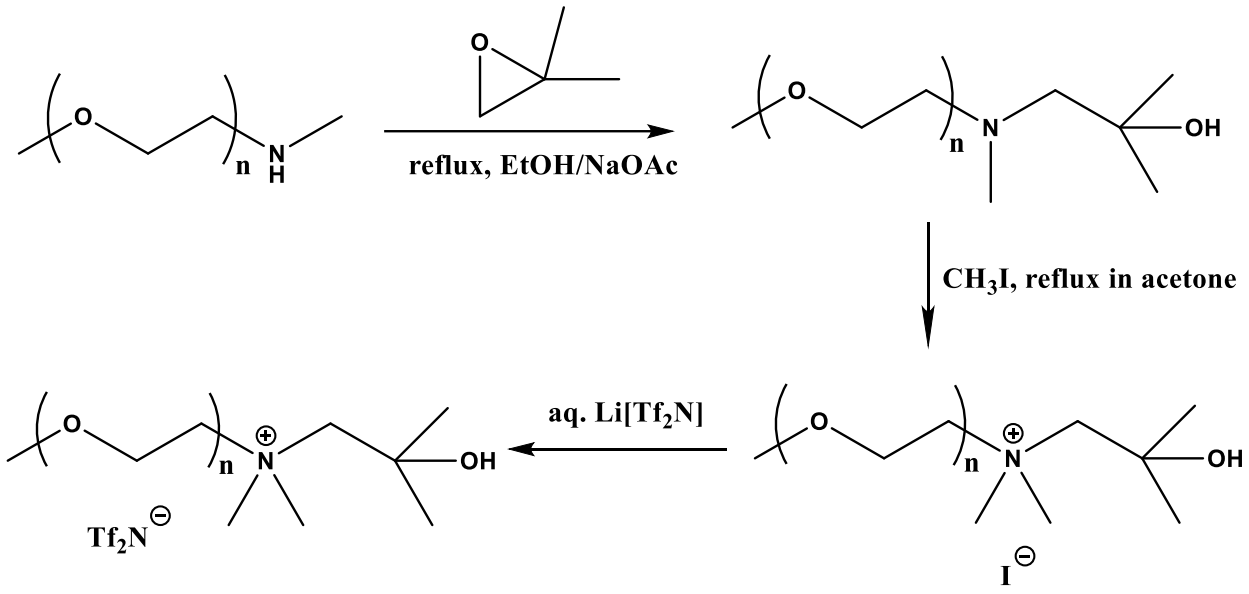

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407 **Scheme 1.** Three-step synthesis of "water-like" ammonium-type ionic liquids (ILs).

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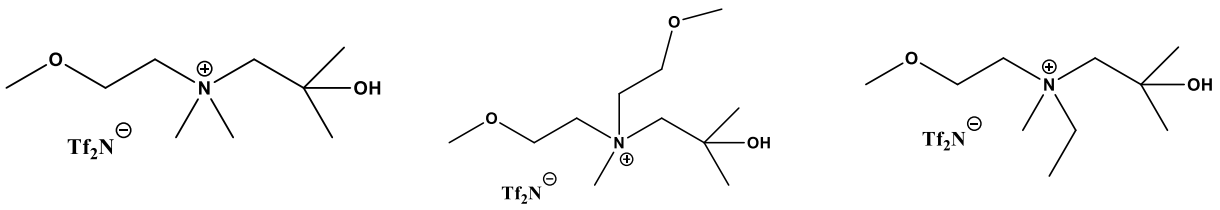

1 **2** **3**

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411 **Scheme 2.** Structures of three dual-functionalized ammonium-based ILs.

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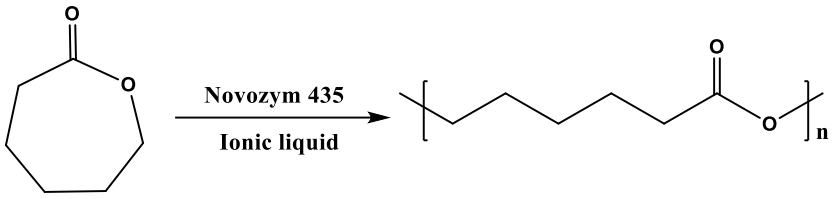

413

414 **Scheme 3**. Enzymatic ring-opening polymerization (ROP) of ε-caprolactone.

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Table 1 Lipase-catalyzed transesterification between ethyl sorbate and 1-propanol [a]

Trial	Solvent (water, wt%) [b]	Dynamic viscosity at 30 °C (mPa s) [c]	Kinematic viscosity ($mm^2 s^{-1}$) [c]	Density at 30 °C ($g\ cm^{-3}$) [c]	Enzyme activity ($\mu mol\ min^{-1}\ g^{-1}$ free CALB) [d]
1	*tert*-Butanol (0.02)	4.31 (25 °C) [65]	–	0.7887 (20 °C) [65]	5.94
2	Diisopropyl ether (0.02)	0.299 [66]	–	0.713 [66]	8.57
3	[BMIM][Tf_2N] (0.01)	41.4	28.9	1.430	5.12
4	[$CH_3OCH_2CH_2$-Im-*t*-BuOH][Tf_2N] (0.02)	303.0	213.3	1.421	**12.36**
5a	[$CH_3OCH_2CH_2$-Me_2N-*t*-BuOH][Tf_2N] (0.005)	**129.3**	92.1	1.403	6.61
5b	[$CH_3OCH_2CH_2$-Me_2N-*t*-BuOH][Tf_2N] (0.01)				7.74
5c	[$CH_3OCH_2CH_2$-Me_2N-*t*-BuOH][Tf_2N] (0.02)				**9.15**
5d	[$CH_3OCH_2CH_2$-Me_2N-*t*-BuOH][Tf_2N] (0.03)				8.02
6a	[$(CH_3OCH_2CH_2)_2$-MeN-*t*-BuOH][Tf_2N] (0.003)	190.4	138.2	1.378	5.81
6b	[$(CH_3OCH_2CH_2)_2$-MeN-*t*-BuOH][Tf_2N] (0.02)				6.73
7a	[$CH_3OCH_2CH_2$-Me-EtN-*t*-BuOH][Tf_2N] (0.002)	175.3	126.7	1.384	4.42
7b	[$CH_3OCH_2CH_2$-Me-EtN-*t*-BuOH][Tf_2N] (0.02)				7.37

Note: [a] The transesterification was conducted by adding ethyl sorbate (5 mM) and 1-propanol (0.67 M) in 1.0 mL solvent with the presence of 20 mg Novozym 435 (~ 4 mg free CALB) at 50 °C. [b] A coulometric Karl Fischer titrator was used to measure the water content at 22 °C with Hydranal® Coulomat AG as the analyte. [c] An Anton Paar SVM 3000 viscometer was used to determine the dynamic/kinematic viscosity and density data at 30 °C (except noted otherwise). [d] The enzyme activity was calculated based on ~4 mg free CALB in 20 mg Novozym 435 (see *Section 2.2* for explanation).

Table 2 Enzymatic ROP of ε-caprolactone using different co-solvents [a]

Trial	Solvent (water content)	Isolated yield (%)	M_w (Da) [d]	PDI
1	no solvent	37	13,800	1.71
2	$[CH_3OCH_2CH_2$-Im-Et$][Tf_2N]$ (0.03 wt%) [b]	42	12,300	1.60
3	$[CH_3OCH_2CH_2$-$Et_3N][Tf_2N]$ (0.03 wt%) [b]	11	17,300	1.39
4	$[CH_3OCH_2CH_2$-Im-*t*-BuOH$][Tf_2N]$ (0.013 wt%) [c]	76	15,900	1.66
5	$[CH_3OCH_2CH_2$-Me_2N-*t*-BuOH$][Tf_2N]$ (0.017 wt%)	74	18,000	1.55
6	$[(CH_3OCH_2CH_2)_2$-MeN-*t*-BuOH$][Tf_2N]$ (0.015 wt%)	64	15,800	1.57
7	$[CH_3OCH_2CH_2$-Me-EtN-*t*-BuOH$][Tf_2N]$ (0.021 wt%)	64	17,600	1.55

Note: [a] General reaction conditions (unless noted otherwise): 0.5 g of ε-caprolactone (containing 0.02 wt% water), 0.25 mL solvent, 100 mg of Novozym 435 (Lot # SLBW1544), gentle stirring (210 rpm) at 70 °C for 2 days. GPC-derived M_w values were based on results calibrated using polystyrene standards. [b] Data (using Novozym 435 Lot # SLBP0766V) were published in our earlier paper [34]. [c] This IL was prepared by an earlier study [39]. [d] Based on GPC analysis.

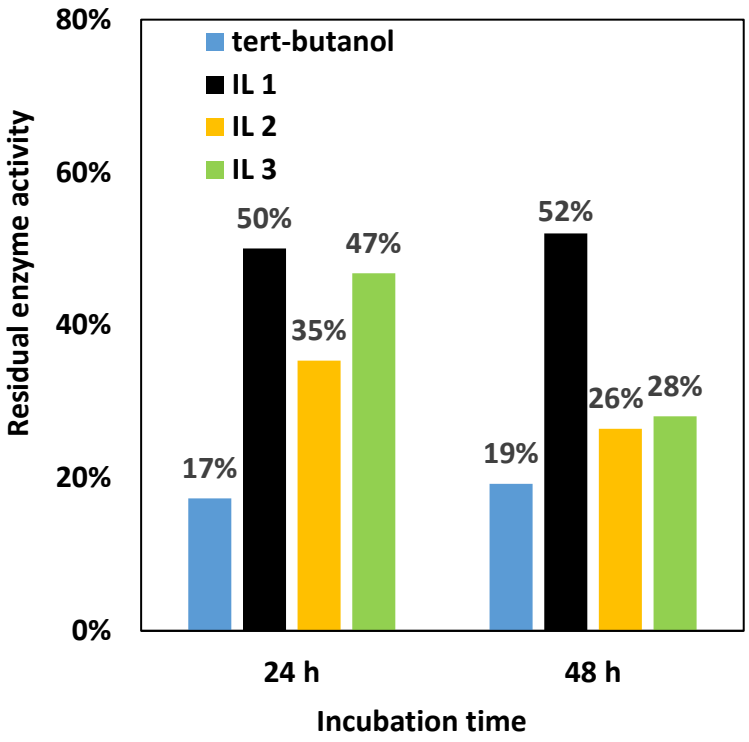

Fig. 1. Novozym 435 thermal stability in various solvents (IL **1**, **2** and **3** structures are shown in Scheme 2). Reaction conditions: a closed vial containing 20 mg Novozym 435 and 1.0 mL solvent was placed in a 50 ºC-oil bath for 24 or 48 h under gentle agitation. At the end of incubation, the mixture was cooled to room temperature, followed by the addition of ethyl sorbate (50 µL 100 mM in 1-propanol). The reaction mixture was sealed and stirred in an oil bath at 50 °C. The lipase activity was determined following the procedure in *Section 2.2*.