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ARTICLE

Highly selective biocatalytic synthesis of monoacylglycerides in Sponge-Like Ionic Liquids

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The biocatalytic synthesis of monoacylglycerides (MAGs) was carried out by the direct esterification of fatty acids (*i.e.* capric, lauric, myristic, palmitic and oleic acids, respectively) with glycerol in different ionic liquids (ILs) based on cations with long alkyl side-chains (*e.g.* 1-hexadecyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide [C_{16}mim][NTf_2], 1-dodecyl-3-methylimidazolium tetrafluoroborate [C_{12}mim][BF_4], etc.). Although all ILs have been shown as suitable reaction media for Novozym 435-catalyzed esterification of glycerol with free fatty acids, a high selectivity of MAGs was only observed in the [C_{12}mim][BF_4] case (*e.g.* up to 100% selectivity and 100% yield for monolaurin). Furthermore, as these ILs are temperature switchable ionic liquid/solid phases that behave as sponge-like systems, a straightforward protocol for IL-free MAGs recovery, based on iterative centrifugations at controlled temperature, has been developed.

1. Introduction

Monoacylglycerides (MAGs) are non-ionic emulsifiers, widely used in food, pharmaceutical, and cosmetic industries.[1] Besides their bulk applications in food and dairy industries, pure MAGs are also of great interest in medicinal chemistry, due to their biological activity (*e.g.* antimicrobial,[2a] to prevent prostatic hyperplasias,[2b] etc.), as well as in the pharmaceutical industry as drug carriers.[3] On the other side, the exponential growth of biodiesel industries all around the world is producing large amounts of glycerol as a by-product, which must be valorised for the sustainability of the biodiesel industry.[4] The use of glycerol for producing MAGs may clearly be one of such valuable targets.[1,5] Today, commercial MAGs are manufactured by chemical glycerolysis of fats/oils and glycerol at high temperatures (220-250ºC), using inorganic alkaline catalysts. The use of high temperature has some drawbacks, such as a dark color, burnt taste, and high energy consumption. Furthermore, this chemical glycerolysis usually provides 35–60% MAGs, 35–50% diacylglycerides (DAGs), 1-20% triacylglycerides (TAGs), 1–10% free fatty acids (FFAs), along with the alkali metal salts.[6] Usually, molecular distillation technique was used to purify the reaction products in order to obtain MAGs of at least 70% purity, to agree with the World Health Organization and the EU directives.[7]

The direct esterification of glycerol with FFAs is one the most popular approaches assayed for carrying out the selective synthesis of MAGs. In this way, both chemical (*e.g.* rationally designed mesoporous siliceous structures containing acidic active sites,[8] etc.) and enzymatic (*e.g.* immobilized lipases)[9] catalysts have been studied. For mesoporous catalysts, for which reaction takes place at high temperatures (100-240ºC), the best selective synthesis of MAGs (up to 96%) was obtained at low conversion levels (5.8-31%).[8a] The increase in conversion level up to 92% was accompanied by a decrease in MAG selectivity up to 62%.[8b] Similar results were obtained by using immobilized lipase as catalysts. As an example, the Lipozyme IM20-catalysed synthesis of monolaurin by esterification of glycerol with lauric acid in a solvent-free system resulted in a 75% conversion and a selectivity towards monolaurin up to 40%.[9a]

From a thermodynamics point of view, an increase in the maximum MAGs content, relative to the total amount of acylglycerides synthesized, could be reached by using a

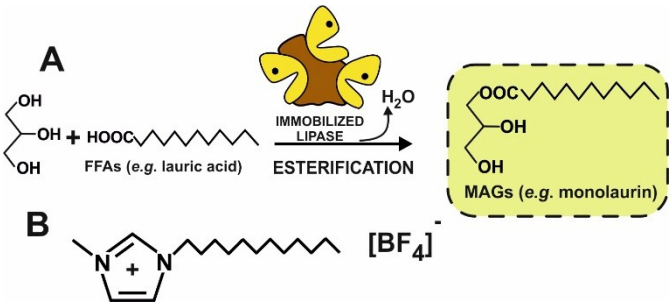

Fig. 1. **A.** Scheme of the immobilized lipase-catalysed synthesis of monoacylglycerides (MAGs) by direct esterification of FFAs (*e.g.* lauric acid with glycerol). **B**. Structure of the [C_{12}mim][[BF_4], as an example of temperature switchable ionic liquid/solid phase used for the selective synthesis of MAGs.

substantial excess of glycerol in the reaction medium. However, the limited solubility of glycerol in TAGs and/or FFAs leads to the formation of biphasic systems with low efficiency for shifting the reaction equilibrium towards an increased MAGs formation. Several strategies based on reaction medium engineering, such as the use of organic cosolvents (*e.g*. *t*-pentanol, *t*-butanol, etc.),[6] or amphoteric surfactants (*e.g.* cocamidopropyl betaine CAPB)[9b] have been assayed in order to provide monophasic reaction media. Through these approaches, conversion yields close to 100 % can be reached, although the selectivity for the MAGs synthesis was similar to previous approaches (*e.g.* Novozym 435-catalyzed glycerolysis of trilaurin in 500% v/v *t*-pentanol resulted in 76.4% MAGs, 14.5% DAGs and 9.1% TAGs after 2 h reaction at 50ºC).[6] In comparison to *t*-butanol, the addition of CAPB leads to similar MAGs contents for enzymatic synthesis of monolaurin, although this surfactant does not need to be removed from the reaction mixture, because it is an approved additive in cosmetic products.[9b]

Ionic liquids (ILs) are exceptional non-aqueous reaction media for carrying out both chemocatalytic[10] and biocatalytic processes.[11] They are liquids, at temperatures lower than 100ºC, which are composed entirely by ions, and their use has led to a green chemical revolution because of their unique array of physical-chemical properties (*i.e*. low vapour pressure, non-flammable nature, high ionic conductivity, good dissolution power towards many substrates, high thermal and chemical stabilities, etc.).[12] Concerning the biocatalytic synthesis of MAGs in ILs, it was reported how Novozym 435 was able to obtain up to 90% yield of MAGs and nearly 100% conversion of triacylglycerides for glycerolysis of commercial oils when using the cocosalkylpentaethoximethylammonium methosulfate [CPMA][MS] IL as the reaction medium.[13] The amphiphilic structure of this water-miscible IL was suggested to be capable of creating a compatible system for glycerol, oils and fats. The simultaneous existence of a hydrophobic long alkyl side-chain containing hydrophilic moieties in the cation, was found to be essential for dissolving triacylglycerides (TAG), as well as for inducing the shift of reaction equilibrium towards the formation of MAGs, although strategies for the recovery of the MAGs product were not reported.[13c]

Although the combination of biocatalysts with ILs has resulted in synergic platforms for many synthetic processes,[11] the development of easy, cheap and/or sustainable approaches for product extraction is key for fully implement this technology.[11e] The green character of ILs as solvents/reaction media should always be demonstrated by their efficient recovery and reuse. In this context, [14] it has been reported how ILs based on cations with long alkyl side-chains, (*e.g*. 1-ocadecyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide, [C_{18}mim][NTf_2], Sponge-Like Ionic Liquids, SLILs) behave as temperature switchable ionic liquid/solid phases, that show an excellent suitability as reaction media for biocatalytic synthetic reactions (*i.e.* synthesis of biodiesel,[15a] terpene ester,[15b]. anysil acetate,[15c] etc.). These ILs are able to dissolve the substrates, and forming monophasic liquid systems upon heating above their melting points (at moderate temperatures). After reaction, it was also observed how these fully clear solutions became solid by cooling down to room temperature. Then, the solid mixture can be separated into two phases by simple centrifugation at a temperature below room. This resulted in an upper liquid phase of nearly pure product, while the bottom phase was the solid IL.[16] In the same context, it was also reported how the mixture of ILs based on imidazolium cations (*e.g*. 1-butyl-3-methylimidazolium,[17a] 1-octyl-3-methylimidazolium,[17b] etc) and the [BF_4] anion, with water behaves as a thermo-responsive system that undergoes a reversible two phase-single phase transformation dependent upon temperature. This property has been successfully applied to carry out selective organometallic transformations *(i.e*. hydrodimerization,[17a] hydrogenation,[17b] etc.) in monophasic IL/water systems resulted by heating, allowing then the easy recovery of products by simple cooling, because of the formation of two-phase liquid systems.

This paper shows by first time a clean protocol for producing nearly pure MAGs, based on the combination of the high selectivity of Novozym-435 for ester synthesis with the unique properties of SLILs, allowing a straightforward separation of the product, as well as the full recovery of the SLIL-biocatalyst system for reuse. The suitability of different SLILs (*e.g.* 1-hexadecyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide [C_{16}mim][NTf_2], 1-dodecyl-3-methylimidazolium tetrafluoroborate [C_{12}mim][BF_4], etc.) as reaction media for Novozym 435-catalysed MAGs synthesis by direct esterification of glycerol with different FFAs (*i.e.* oleic, palmitic, myristic, lauric and capric acids) has been demonstrated. The optimization of both the reaction conditions (*e.g*. FFA-glycerol molar ratio, nature of the SLILs, etc.), as well as the cooling/centrifugation protocol for products separation, based on the sponge-like behavior of these ILs, are shown.

2. Experimental

2.1 Chemicals

Immobilized *Candida antarctica* lipase B (Novozym® 435, EC 3.1.1.3) was obtained from Novozymes S.A. (Spain). Glycerol 99% purity), capric acid (98% purity), lauric acid (98% purity), myristic acid (99% purity) palmitic acid (99% purity), oleic acid (99% purity), anhydrous *tert*-butanol (99.5% purity), molecular sieves 13X (MS13X; 10 Å pore size, 270 mg H_2O/g adsorption capacity), solvents and other chemicals were obtained from Sigma-Aldrich-Fluka (Madrid, Spain). The ILs 1-octadecyl-3-methylimidazolium bis(trifluoromethylsulfonyl) imide ([C_{18}mim][NTf_2], 99% purity) 1-hexadecyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide ([C_{16}mim] [NTf_2], 99% purity), 1-tetradecyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide ([C_{14}mim][NTf_2], 99% purity), 1-dodecyl-3-methylimidazolium bis(trifluoromethyl-sulfonyl)imide ([C_{12}mim][NTf_2], 99% purity), 1-decyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide ([C_{10}mim][NTf_2], 99% purity), 1-hexadecyl-3-methylimidazolium tetrafluoroborate ([C_{16}mim][BF_4], 99% purity) 1-tetradecyl-3-methylimidazolium

tetrafluoroborate ([C_{14}mim][BF_4], 99% purity) and 1-dodecyl-3-methylimidazolium tetrafluoroborate ([C_{12}mim][BF_4], 99% purity), were obtained from IoLiTec GmbH (Germany).

2.2 Lipase-catalyzed esterification of glycerol with free fatty acids in SLILs

Into 3-mL screw-capped vials with teflon-lined septa, 2, 0.7 or 0.3 mmol of capric acid, lauric acid, myristic acid, palmitic acid or oleic acid, respectively, were mixed with 2 mmol of *t*-butanol and the corresponding amount of glycerol to finally achieve a 1:4 acid:glycerol molar ratio. Then, the corresponding amount of IL ([C_{10}mim][NTf_2], [C_{12}mim][NTf_2], [C_{14}mim][NTf_2], [C_{16}mim][NTf_2], [C_{18}mim][NTf_2], [C_{12}mim][BF_4], [C_{14}mim][BF_4] or [C_{16}mim][BF_4], respectively) was added to reach a 45% (w/w) IL final concentration with respect to the overall mass. The resulting reaction mixtures were pre-incubated at 60ºC for 10 min, leading to monophasic systems, and then 240 mg MS13x were also added. The reaction was started by adding Novozym 435 (60 mg per mmol of carboxylic acid) and the reaction mixture was shaken (200 rpm) at 60ºC for 8 h under vacuum conditions. To obtain time-course profiles, 15 µL aliquots were taken at regular intervals and suspended in 485 µL of a dodecane/isopropanol (95:5, v/v) solution, and the resulting biphasic mixtures were strongly shaken for 3 min, and then centrifuged at 15,000 rpm for 15 min at 6ºC to precipitate the IL. Finally, 300 µL of the dodecane/isopropanol liquid phase (upper phase containing acylglycerides) were added to 200 µL of 10 mM tributyrin (internal standard) solution in dodecane/isopropanol (95:5, v/v), and the final solution was analysed by CG.

2.3 GC analysis

GC analysis was performed with a Shimadzu GC-2010 (Shimadzu Europe, Germany) equipped with an FID detector and an automatic injector. Samples were analysed on a TRB-BIODIESEL capillary column (10 m x 0.28 mm x 0.1 µm, Teknokroma, Spain), using tributyrin as internal standard, under the following conditions: carrier gas: He at 30.0 kPa (15 mL/min total flow); temperature programme: 100ºC, 10ºC/min, 200 ºC, 15 ºC/min, 370 ºC, variable split ratio (80:1 to 10:1); temperature for the detector, 370ºC.[15] Peak retention times (min) were as follows: tributyrin, 5.5; capric acid, 2.1; lauric acid, 3.5; myristic acid, 5.4; palmitic acid, 6.8; oleic acid, 8.2; monocaprin, 5.8; monolaurin, 7.5; dilaurin, 14.4; trilaurin, 18.9, monomyristin, 9.0; monopalmitin, 10.4; monoolein, 11.6; diolein, 19.8.

2.4 Recovery of products from the SLILs

For the case of SLILs based on [NTf_2] anion, the monoolein/[C_{18}mim][NTf_2] reaction mixture can be taken as a representative example. This reaction mixture was placed into a 2-mL vial, and then incubated at 60ºC until a fully clear and homogeneous phase was observed. Then, hot water (1 mL, 60ºC) was added, and the resulting multiphase solution was strongly shaken for 30 min at 60ºC, being finally cooled to room temperature. The monoolein/water/SLIL multiphasic mixture was consecutively centrifuged three times at 15,000 rpm (60 min) and at room temperature (not-controlled), 23 and 15ºC, respectively, resulting in three phases, as follows: a top phase of acylglycerides, an aqueous middle phase and a bottom phase containing the solid IL. The top phase was collected, washed again with 1 mL of water at 60ºC for 15 min, and finally centrifuged (30 min at room temperature) to reach a full separation between monoolein and the SLIL. The resulting clean acylglyceride fraction (top phase) was collected, and the SLIL content was determined by ^{19}F NMR. For this, an 80 µL sample of top phase dissolved in 450 µL acetone-δ_6 containing TFA (80 µL), as internal standard, was prepared. Samples were analysed by 300 MHz ^{19}F NMR in a Brucker AC 200E spectrometer, and the residual IL was quantified with respect to a standard [C_{18}mim][NTf_2] solution in acetone-δ_6 containing TFA.

For the case of SLILs based on the [BF_4] anion, each acylglycerides/[C_{12}mim][BF_4] reaction mixture was placed into a 2-mL vial, and then incubated at 60 ºC until a fully clear and homogeneous phase was observed. Then, dodecane (1 mL) was added to each sample, and the resulting fully clear monophasic solutions were strongly shaken for 3 min at room temperature and finally incubated into an ice-bath for 15 min. The acylglycerides/SLIL/dodecane mixture was centrifuged at 15,000 rpm (15 min) and at 6ºC, resulting in the full precipitation of [C_{12}mim][BF_4]. The top phases was collected, and the residual IL content was analysed by ^{19}F NMR, as described above by using a [C_{12}mim][BF_4] solution in acetone-δ_6 containing TFA, as the standard (see ESI). For the separation of acylglycerides from dodecane, the top phases were incubated for 5 min at -10ºC, and then centrifuged at 15,000 rpm (15 min) and at 0ºC, resulting in the precipitation of acylglycerides.

2.5 Identification of acylglycerides by GC/MS

GC-MS analyses of reaction media were carried out by using a GC-6890 (Agilent, USA) instrument coupled with a MS-5973 (Agilent, USA) system. The GC was equipped with an HP-5MS column (30 m × 0.25 mm × 0.25 µm, Agilent, USA). The following conditions were used: carrier gas: He at 1 mL/min; inlet split ratio: 1:1; temperature program: 60°C, 1 min; 10°C/min, 300°C, 5 min; MS source ionization energy, 70 eV; the scan time was 0.5 s, covering a mass range of 40–800 amu. Each FAMEs and FASEs peak was identified by comparison of their mass spectra with those in a computer library (NIST Library). Monocaprin, retention time (Rt, min): 16.8; positive ion (m/z): 57.1, 74.1, 98.1, 134.1, 155.1, 173.1, 215.2. monolaurin, Rt: 18.5; (m/z): 57.1, 74.1, 98.1, 117.2, 134.1, 157.1, 183.2, 201.2, 243.2. Dilaurin, Rt: 22.7; (m/z): 57.1, 74.1, 98.1, 117.1, 134.0, 183.2, 201.2, 243.2. Trilaurin, Rt: 27.0; (m/z): 57.1, 74.1, 98.1, 129.1, 183.2, 243.2. Monomyristin, Rt: 17.5; (m/z): 57.1, 73.1, 97.1, 134.1, 173.1, 199.2, 229.2. Monopalmitin, Rt: 19.0; (m/z): 57.1, 74.1, 97.1, 129.1, 173.1, 199.2, 239.3, 257.3. Monoolein, Rt: 20.6; (m/z): 55.1, 97.1, 123.1, 151.1, 180.2, 222.2, 265.3, 338.3. Diolein, Rt: 23.4; (m/z): 55.1, 74.1, 101.1, 131.1, 152.1, 172.1, 203.1, 264.3, 339.3.

3. Results and discussion

3.1 Lipase-catalysed monoacylglycerides synthesis by direct esterification of glycerol

The suitability of immobilized *Candida antarctica* lipase B for carrying out the biocatalytic synthesis of MAGs by direct esterification of FFAs (*i.e.* capric acid, lauric acid, myristic acid, palmitic acid and oleic acid) with glycerol was studied in eight different SLILs (*i.e.* $[C_{10}mim][NTf_2]$, $[C_{12}mim][NTf_2]$, $[C_{14}mim][NTf_2]$, $[C_{16}mim][NTf_2]$, $[C_{18}mim][NTf_2]$, $[C_{12}mim][BF_4]$, $[C_{14}mim][BF_4]$ and $[C_{16}mim][BF_4]$),as reaction media at 60ºC. As a representative example, Fig. 2 depicts the time-course profiles for the enzymatic esterification of lauric acid with glycerol in $[C_{12}mim][NTf_2]$ (Fig. 2A), $[C_{16}mim][NTf_2]$ (Fig. 2B) and $[C_{12}mim][BF_4]$ (Fig. 2C) ILs at 60ºC. As can be seen in Fig 2A, the immobilized enzyme provided a full consumption of the lauric acid substrate, leading to a final mixture of both monolaurin (ca. 85%) and dilaurin (ca. 15%) in $[C_{12}mim][NTf_2]$ reaction medium, which was maintained constant for 8 h reaction. Taking into account that the esterification reaction is thermodynamically controlled, the presence of molecular sieves, as a dehydrating agent, as well as the use of vacuum conditions, are fully involved in the overall shift of the reaction equilibrium towards the esterification products, because of the elimination of water by-product.[15b] However, as the enzyme is able to catalyse esterification of one, two or all the three hydroxyl groups in the glycerol molecule (producing MAG, DAG or TAGs, respectively), the selective synthesis of MAGs is probably related with the mass-transfer phenomena (diffusion) around the active site of the enzyme, being determined by the characteristic of the reaction medium. In this context, the presence of minor amount of *t*-butanol as polar cosolvent aids to reduce viscosity of the medium favouring mass-transfer, but the use of a hydrophobic IL covering enzyme particles may be a difficulty for the diffusion of the hydrophilic glycerol molecules to the enzyme active site, favouring the multi-esterification of the glycerol molecule. This last factor could explain the observed loss in the selectivity (concentration of the MAG with respect to all the acylglycerides formed) towards the desired monolaurin product (see Fig 2A).

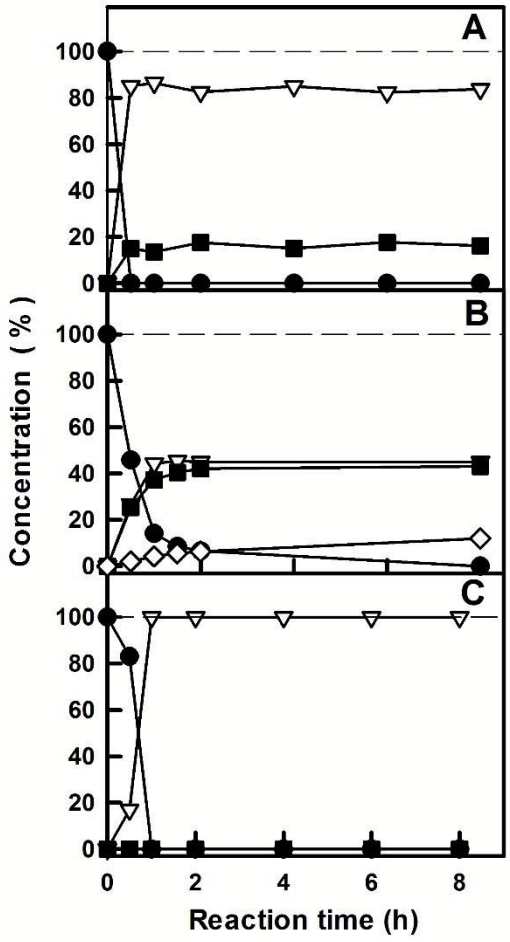

Fig. 2. Time-course profiles of lauric acid (●), monolaurin (▽), dilaurin (■) and trilaurin (◇) for the Novozym 435-catalysed esterification of lauric acid with glycerol in $[C_{12}mim][NTf_2]$ (**A**), $[C_{16}mim][NTf_2]$ (B) and $[C_{12}mim][BF_4]$ at 60ºC.

Table 1. Biocatalytic synthesis of acylglycerides by esterification of FFAs with glycerol in different ionic liquids after 4 h of reaction at 60ºC. See Experimental section for further details).

Entry	IL	FFA (mmol)	Conv. (%)	MAG (%)	DAG (%)	TAG (%)
1	None	2.0[b]	98	65	29	6
2	None	2.0[e]	77	59	41	0
3	$[C_{10}mim][NTf_2]$	2.0[b]	91	62	33	5
4	$[C_{10}mim][NTf_2]$	2.0[e]	80	62	38	0
5	$[C_{12}mim][NTf_2]$	2.0[b]	100	85	15	0
6	$[C_{14}mim][NTf_2]$	2.0[e]	65	55	45	0
7	$[C_{16}mim][NTf_2]$	0.7[b]	93	48	45	7
8	$[C_{16}mim][NTf_2]$	2.0[e]	67	58	42	0
9	$[C_{18}mim][NTf_2]$	2.0[b]	91	68	26	6
10	$[C_{18}mim][NTf_2]$	2.0[e]	74	66	34	0
11	$[C_{12}mim][BF_4]$	0.7[b]	66	100	0	0
12	$[C_{12}mim][BF_4]$	0.3[a]	100	100	0	0
13	$[C_{12}mim][BF_4]$	0.3[b]	100	100	0	0
14	$[C_{12}mim][BF_4]$	0.3[c]	100	100	0	0
15	$[C_{12}mim][BF_4]$	0.3[d]	80	100	0	0
16	$[C_{12}mim][BF_4]$	0.3[e]	53	100	0	0
17	$[C_{14}mim][BF_4]$	0.3[e]	39	100	0	0
18	$[C_{16}mim][BF_4]$	0.3[e]	11	100	0	0

FFAs: [a]capric acid; [b]lauric acid; [c]myristic acid; [d]palmitic acid; [e]oleic acid.

This hypothesis was confirmed when the [C_{16}mim][NTf_2] IL was used as reaction medium (see Fig 2B). For this IL, although the enzyme also provides a full transformation of lauric acid into ester products, the increase in the alkyl-side chain length of the imidazolium cation, caused a loss in the selectivity for monolaurin synthesis relative to [C_{12}mim][NTf_2] (*i.e.* 48% monolaurin, 45% dilaurin and 7% trilaurin), in spite of using lower FFA/glycerol ratio that should favour selectivity towards the MAGs. In order to favour the transport of glycerol molecules towards the enzyme particles by improving the hydrophilicity of the biocatalyst microenvironment, the [C_{12}mim][BF_4] IL was also tested as reaction medium (see Fig. 2C). For this case, the enzyme was also able to provide the full transformation of lauric acid substrate to only one esterification product (MAG), being the selective synthesis of monolaurin fully reached after 2 h reaction and maintained unchanged after 8 h (see Fig. 2C). The unique characteristics of this IL, based on a hydrophobic cation (*i.e.* [C_{12}mim]) and the water-miscible [BF_4] anion, provides an appropriate amphiphilic ionic net as reaction medium, that permits the transport of both lauric acid and glycerol substrates (which are mutually immiscible) to the enzyme active site, as well as the exit of the formed MAGs product to the bulk reaction medium.

Table 1 shows the FFA conversion and the yields for the different acylglyceride products resulting from the direct esterification of capric acid, lauric acid, myristic acid, palmitic acid or oleic acid, respectively, with glycerol in eight different SLILs after 4 h of reaction at 60ºC. Although the enzyme was able to catalyse the esterification reaction in the absence of ILs (entries 1 and 2), the selectivity towards the MAGs synthesis was weak. The presence of hydrophobic ILs based on long alkyl chains in the imidazolium cation (see entries 3 to 10) did not provide improvements in the selectivity to MAGs synthesis in most cases. The formation of appreciable amounts of DAGs was concomitantly observed.

Changing the [NTf_2] anion by the less hydrophobic [BF_4] anion, and maintaining the [C_{12}mim] cation, the selective synthesis of MAGs was clearly enhanced, though the conversion was reduced (entry 11), requiring the use of lower FFA/glycerol molar ratios (entries 12-18). Quantitative conversions and full selectivities were obtained using the [C_{12}mim][BF_4] IL as reaction media for the capric, lauric, myristic, palmitic and oleic acids at a 0.3 FFA/glycerol molar ratio (entries 12-14). For palmitic acid, the conversion was slightly lower (entry 15), while for the oleic acid, a moderate conversion was achieved after 4 h of reaction time (*i.e.* 53%, entry 16). The use of [C_{14}mim][BF_4] and [C_{16}mim][BF_4] ILs (see entries 17 and 18) did not improve the results in oleic acid conversion, most likely because of the resulting semisolid and highly viscous reaction media, although the selectivity towards MAGs synthesis was always 100%. These results clearly show the excellent suitability of ILs based on cation containing long alkyl chain and the [BF_4] anion for the selective biocatalytic synthesis of MAGs with independence of the nature of the FFA.

ILs have been defined as nano-structured reaction media that allow hydrophobic molecules to reside in less polar regions, and polar species (*i.e.* methanol) to undergo faster diffusion in the more polar regions.[10,14] The aliphatic chain of the cation provides an appropriate environment for the mass transfer of FFAs to the enzyme active site, while the less hydrophobic nature of the [BF_4] anion can facilitate the transport of glycerol (acyl acceptor) to the enzyme microenvironment. Thus, after the first esterification reaction takes place onto a glycerol molecule, the MAG product can be easily transported to the macroenvironment, which results in the excellent selectivity towards MAG synthesis. On the contrary, by using more hydrophobic ILs, the transport of glycerol molecules (acting as acyl acceptor) to the enzyme microenvironment is reduced. In these conditions, the MAG product is re-used by the enzyme as an acyl acceptor for a second esterification reaction, resulting in the DAGs synthesis. In this context, it worth mentioning that the suitability of the water-miscible [CPMA][MS] for improving the enzymatic synthesis of MAGs by glycerolysis of was related to its amphiphilic structure, being key for dissolving both hydrophobic (*i.e.* TAGs) and hydrophilic (*i.e.* glycerol) substrates and favouring the equilibrium shift towards synthetic product.[13] In our case, the hydrophobic [C_{12}mim] cation and the water-miscible [BF_4] anion provide appropriate environment for dissolving both FFAs and glycerol, respectively.

Biocatalytic cyclic production of MAGs in sponge-like ILs.

Two milestones need to be achieved for scaling-up any biocatalytic process for MAGs production in ILs: the high activity and operational stability in the biocatalyst, and the easy separation of products by a clean approach, including the recovery of the IL for reuse. Figure 3 shows the operational stability profile of the Novozym 435 biocatalyst against operation cycles of reuse for both [C_{12}mim][NTf_2] (Fig. 3A) and [C_{12}mim][BF_4] (Fig. 3.B) as reaction media at 60ºC. As can be seen, the MAG yields and selectivities (for lauric acid case) were remained unchanged after 8 consecutive cycles of reuse in both cases. The excellent ability of ILs to over-stabilize enzymes in non-aqueous conditions for continuous reuse has been widely reported,[14,15,18,19,21] even under extremely harsh conditions (*i.e.* $scCO_2$ at 120 bar and at 150ºC).[18b] In this context, ILs systems based on cations with a long alkyl chain were excellent for preserving the catalytic activity of the enzyme, *i.e.* half-lives of up to 1370 d under operational conditions during biodiesel synthesis.[15a] Furthermore, it was also reported how this extremely ordered supramolecular structure of ILs in liquid phase might also be able to act as a ‘‘mould’’, stabilizing the active 3-D structure of the enzyme in these non-aqueous nano-environments.[14,19]

As mentioned above, the development of selective transformation and separation processes, able to directly provide pure products and the full recovery of solvents and catalysts, is a key target in green chemistry. The unique properties of ILs based on cations with long alkyl side-chains, (*e.g.* ([C_{18}mim][NTf_2], etc.), as temperature switchable ionic liquid/solid phases behaving as sponge-like systems (SLILs), has recently been applied to successfully achieve these goals in the biocatalytic synthesis of terpene esters,[15b,c] biodiesel,[15a] etc.).

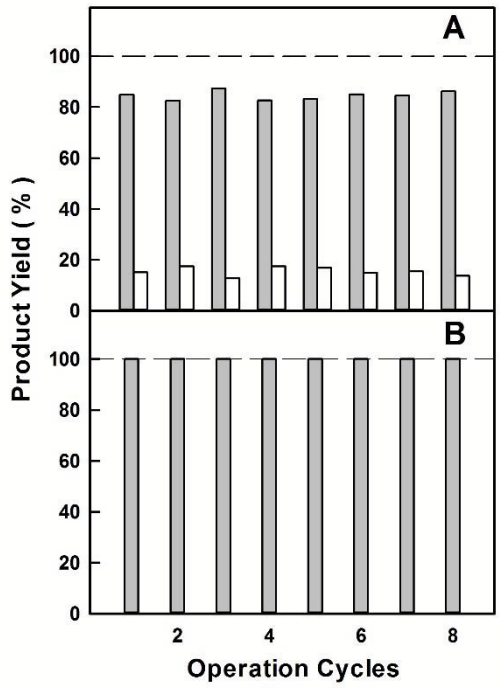

Fig. 3. MAGs (grey) and DAGs (white) product yields during continuous operation cycles for the Novozym 435-catalyzed esterification of lauric acid with glycerol in [C_{12}mim][NTf_2] (**A**) and [C_{12}mim][BF_4] (**B**)at 60ºC.

Taking into account the melting points of the assayed ILs in Table 1,[20] an easy protocol for acylglyceride extraction was developed, based on the sponge-like behaviour.[14] For this purpose, the reaction media based on [C_{18}mim][NTf_2] (see entry 10) and ([C_{12}mim][BF_4] (entry 13) were selected as representative examples for developing an efficient MAGs separation. As can be seen in Fig. 4A, the monoolein/[C_{18}mim][NTf_2] reaction medium is a fully clear monophasic systems at 60ºC, which became solid after cooling to room temperature. The addition of water resulted in a heterogeneous mixture (Fig. 4B). After a vigorous shaking for 30 min at 60ºC, the cooling down to room temperature provided a semisolid heterogeneous mixture. Following an iterative cooling/centrifugation protocol (see experimental section for details), three separated phases were obtained: an upper liquid phase containing monoolein, a middle liquid aqueous phase containing excess of glycerol,[15a] and a bottom solid containing the IL (Fig. 4C). The presence of water, a green molecular solvent non-miscible with monoolein nor [C_{18}mim][NTf_2], improved the separation between phases, which followed the density parameter (monoolein < water < IL). By ^{19}F NMR, a 3 % (w/w) residual IL content in the MAG top phase was determined (see ESI), which could be reduced by additional washing steps with hot water.[15a]

For the monoolein/[C_{12}mim][BF_4] system, the application of the same protocol described above presented some difficulties. Thus, the addition of hot water to this reaction media and the subsequent shaking produced a gel after cooling down, making impossible any separation by centrifugation (see Fig 4D). The

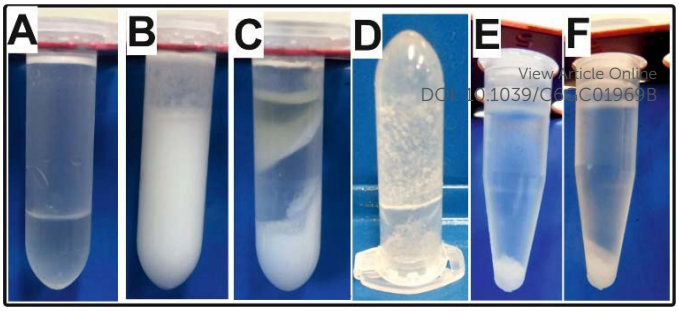

Fig. 4. Phase behaviour of monoolein/[C_{18}mim][NTf_2] reaction mixture (Table 1, entry 10) at 60ºC (**A**), after addition of H_2O at 60ºC (**B**), and after three consecutive centrifugation steps at 15,000 rpm (1 h) and at room temperature, 23 and 15ºC (**C**). Phase behaviour of monolaurin/[C_{12}mim][BF_4] reaction mixture (Table 1, entry 13) after addition of H_2O at 60ºC (**D**), after addition of dodecane (1 mL) and centrifugation at 10ºC (**E**). Picture **F** shows the precipitation of MAGs present in the top-phase (picture **E**) by centrifugation at 0ºC. See experimental section for further details.

addition of polar cosolvents (*e.g.* ethanol, acetone, etc.) did not improve the separation. However, it was observed how this [C_{12}mim][BF_4] was soluble in dodecane at room temperature, but precipitating at lower temperatures. Thus, the monoolein/[C_{12}mim][BF_4] reaction mixture was dissolved in dodecane and then centrifuged at 6ºC, which resulted in the full precipitation of the IL (see Fig. 4E), and an IL-free MAGs/dodecane phase, as it was determined by ^{19}F NMR. This IL-free dodecane phase was also obtained for reaction media show in Table I as entries 12-16 (see ESI). The monoolein product was separated by incubating for 5 min at -10ºC, and then centrifuged at 15,000 rpm (15 min) and at 0ºC, which results in the precipitation of the IL-free MAG (see Fig. 4F), and the recovery of dodecane solvent. As can be seen, the proposed methodology for nearly pure MAGs production also includes a straightforward way to separate and isolate all the component of the reaction mixture, allowing the recovery and reuse of the solvent used including the ILs, by simple centrifugation at controlled temperature.

Conclusions

This paper shows for the first time an easy and green method for the highly selective synthesis of MAGs by direct esterification of FFAs with glycerol by using two key tools for green chemistry, as are biocatalysts and SLILs. Enzymes are the most selective catalysts provided by Nature for chemical transformations in living systems. Ionic liquids, and mainly those based on long alkyl side chain(s) on the cation, are non-aqueous reaction media widely recognised to preserve catalytic properties of enzymes.[14,20] The sponge-like behaviour of some of them, as temperature switchable ionic liquid/solid phases, allows developing excellent reaction media for dissolving FFA and glycerol substrates. A proper selection of the cation and anion structures in these SLILs also allows controlling the mass transfer processes into and from the active site of the enzyme, favouring the achievement of selective synthetic processes for the preparation of MAGs. In this way, the SLILs based on the

[BF_4] anion were shown as exceptional media for the selective synthesis of MAGs (*i.e.* up to 100% selectivity, without any loss in the catalytic activity with the reuse). Their SLIL behaviour facilitates the development of straightforward protocols, based on cooling and centrifugation steps that allow an easy and full recovery of both nearly pure MAGs products and the IL and any other solvent used.

Using this approach, almost pure MAG products were obtained, while recovery and reuse of the biocatalyst/SLIL system led to maintenance in activity in agreement with the demonstrated ability of SLIL to stabilize enzymes. The marriage between enzymes and the considered Sponge-Like Ionic Liquids (SLILs) provides synergic opportunities and opens up the way to new sustainable platforms for developing green chemical processes of industrial interest.

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Graphical abstract: text and Figure:

Highly selective biocatalytic synthesis of monoacylglycerides in Sponge-Like Ionic Liquids

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Monoacylglycerides are biocatalytically synthesized by direct esterification in sponge-like ionic liquids with high selectivity (*e.g.* up to 100% monolaurin in $[C_{12}mim][BF_4]$).