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# *Lipase Catalyzed Epoxy-acid Addition and Transesterification: from Model Molecule Studies to Network Build-up*

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## **Abstract**

Commercially available lipase from *Pseudomonas stutzeri* (Lipase TL) is investigated as biocatalyst for the formation of an acid-epoxy chemical network. Molecular model reactions are performed by reacting 2-phenyl glycidyl ether and hexanoic acid in bulk, varying two parameters: temperature and water content. Characterizations of the formed products by <sup>1</sup>H NMR spectroscopy and GC-MS combined with enzymatic assays confirm that lipase TL is able to simultaneously promote acid-epoxy addition and transesterification reactions below

100°C and solely the acid-epoxy addition after denaturation at  $T > 100^\circ\text{C}$ . A prototype biobased chemical network with  $\beta$ -hydroxyester links was obtained using resorcinol diglycidyl ether and sebacic acid as monomers and the lipase TL as catalyst. DSC, ATR-IR, and swelling analysis confirm gelation of the network.

## Introduction

Among enzymes, lipases are a preferred option as biocatalyst for chemical synthetic applications due to their low cost, high stability (organic solvent, temperature) and high tolerance in substrate chemical structure.<sup>1,2</sup> Mainly used as a transesterification catalyst for biofuel production,<sup>3-5</sup> they are also able to catalyze a wide range of chemical transformations, such as  $\alpha$ -epoxidation,<sup>6,7</sup> acylation,<sup>8</sup> alcoholysis,<sup>9</sup> and aminolysis,<sup>10</sup> - amongst others. Over the past three decades, the combination of rapid development in bio-engineering methodologies and increasing demand for specific properties has led to the production of novel lipases (more widely, enzymes). These enzymes possess enhanced stability (to temperature, pH, organic solvents, *etc.*), selectivity and versatility, which is continuously expanding their range of applications.<sup>11,12</sup>

In synthetic polymer chemistry, lipases have been investigated for monomer production,<sup>13,14</sup> depolymerization reactions,<sup>15</sup> and extensively as a catalyst for linear polyesters production. Synthesis *via* polycondensation of di-acids/di-esters with diols<sup>16,17</sup> and ring-opening polymerization (ROP) of various cyclic esters<sup>18-22</sup> (lactones, lactide, *etc.*) - or the combination of both reactions - have been performed.<sup>23</sup> Polymerizations are commonly carried out in an organic solvent (*e.g.* toluene) at moderate temperature (60-90°C) using supported-lipases (*e.g.* Novozym 435 ®). Less conventional enzyme-catalyzed ROP involving reactive extrusion and higher processing temperature (90-130°) have also been reported for the synthesis of high molar mass polyesters derivatives.<sup>24,25</sup> Apart from ROP and polycondensation, lipases have

shown their ability to catalyze the synthesis of ( $\beta$ -hydroxy) linear polyester derivatives *via* epoxy-acyl polyaddition mechanisms.<sup>26,27</sup> Low molar mass linear polyesters (from 1000 to 13 500 g.mol<sup>-1</sup>) were produced by reacting mono-functional epoxides (glycidyl ester derivatives or styrene oxide) and cyclic anhydrides in similar conditions (60-90°C, bulk or solvent).

Enzymatic methodology transfer towards bulk material production, such as epoxy-acid thermoset, is accompanied by major limitations. One of the main expected limits is related to the reduced segment mobility after gelation and vitrification which may cause incomplete monomer consumption.<sup>28,29</sup> As a breakthrough, the preparation of epoxy-acid crosslinked materials in a fully enzymatic catalysis approach has been described in a recent patent.<sup>30</sup> The authors claim they have obtained chemically crosslinked networks while processing at moderate temperature (100°C) and from a stoichiometric amount of diepoxide (the diglycidyl ether of bisphenol A, DGEBA) and di-acid (sebacic acid) monomers. As all these monomers are bi-functional, the formation of a chemically crosslinked network at (1:1) stoichiometry is impossible if acid-epoxy addition is the sole reaction to occur. Gelation requires branching by transesterification to take place.<sup>31,32</sup> Furthermore, acid curing of epoxides is typically performed at temperature higher than 130°C; <sup>32,33</sup> effective activation of acid-epoxy addition and transesterification at 100°C strongly suggests that both reactions are catalyzed by lipases. Among a wide range of commercially available lipases, the best candidate for the curing process was determined to be lipase from *Pseudomonas stutzeri* (commercially available under the name of Lipase TL by Meito Ltd) for its ability to promote complete curing, efficient reprocessing and thermal stability.<sup>30</sup>

Herein, to clarify the network's formation in this enzymatic approach, we investigate model molecule reactions between 2-phenyl glycidyl ether and hexanoic acid catalyzed by Lipase from *Pseudomonas stutzeri* (Lipase TL) under typical conditions of epoxy curing (bulk, 60-

140°C). Optimized conditions were further employed for the preparation of a prototype bio-based epoxy-acid network.

## **Experimental section**

*Chemicals.* Lipase TL (from Meito, 27 kDa (SDS PAGES), Activity : 650 U.g<sup>-1</sup> (nitrophenol hydrolysis)), Albumin bovine (Sigma Aldrich, 98% (Fraction V)), 2-phenyl glycidyl ether (b.p. 245°C, Alfa, 98%), hexanoic acid (b.p. 205°C, Alfa, 99%+), resorcinol diglycidyl ether (Sigma Aldrich, 99%), sebacic acid (Sigma Aldrich, 99%), triolein (Sigma Aldrich, 99%+) and 2-Phenyl imidazole (Alfa, 99%+) were used as received without further purification.

*Nuclear Magnetic Resonance (NMR) Spectroscopy.* <sup>1</sup>H NMR spectra were recorded at 24°C using a Bruker AVANCE400 spectrometer. Spectra were referenced to the residual solvent peaks. For <sup>1</sup>H NMR: δ (ppm) 7.26 in CDCl<sub>3</sub>

*FT-IR analysis.* IR measurements were recorded with a Bruker TENSOR 37 spectrometer equipped with a Specac golden gate ATR heating cell.

*GC-MS analysis.* Aliquots are taken from the crude mixture at different times, dissolved in ethyl acetate (in concentrations about 1 mg/mL), and analysed with Shimadzu GC-2010 gas chromatograph coupled to mass spectrometry.

*Differential scanning calorimetry (DSC)* was performed at a heating rate of 10 °C.min<sup>-1</sup> under helium using a TA DSC 250 analyser. Two heating ramps were performed from -20 to 150 °C. The glass transition temperature T<sub>g</sub> was determined from the second heating cycle.

*Molecular reaction catalyzed by lipase TL.* In a 5 mL vial, were introduced successively Lipase TL (26.6 mg, 3 w.%), water (2.7 mg, 0.3 w.%) and 2-phenyl glycidyl ether (500 mg, 3.33 mmol). After solubilisation of the enzyme, hexanoic acid (387 mg, 3.33 mmol) was added and led to a partial precipitation of the enzyme. The vial was then sealed and the

reaction mixture heated up to the desired temperature (60-140°C). Aliquots were taken at different times and the crude product was analysed by GC-MS and <sup>1</sup>H NMR (CDCl<sub>3</sub>).

*Reaction with 2-Phenyl imidazole.* In a 5 mL vial, were introduced successively 2-Phenyl imidazole (2-PI) (26.6 mg, 3 w.%) and 2-phenyl glycidyl ether (500 mg, 3.33 mmol). After solubilisation of the catalyst, hexanoic acid (387 mg, 3.33 mmol) were added to the mixture. The vial was then sealed and the reaction mixture heated up to 100°C for 3h. Analysis of the crude product by <sup>1</sup>H NMR (CDCl<sub>3</sub>) is presented Figure S1.

*Enzymatic assays.* Enzyme activity was measured *via* triolein hydrolysis in emulsified medium. The reaction mixture consisted of triolein (0.5 g), Triton X-100 (2.5 g) and distilled water (7 g). The emulsion was obtained by vigorous stirring (1000 rpm) at 45 °C for 10 min. Then, a proper amount of the target sample (commercial enzyme solution, epoxy enzyme mixture or thermally denaturated enzymes) was added and the reaction started. After 15 min of reaction, hydrolysis was quenched by addition of ethanol (20 mL). Enzymatic activity was calculated by determination of liberated oleic acid through titration with KOH (0.0535 M, addition *via* a syringe pump (2.0 mL.min<sup>-1</sup>) of duplicate samples using naphthol-1-phthalein as end-point indicator.

*Chemical network synthesis.* Resorcinol diglycidyl ether (4.00 g, 18.00 mmol), water (20 mg) and lipase (208 mg) were mixed using a stainless-steel spatula for 2 min. Freshly grinded sebacic acid (3.62 g, 18.00 mmol) was incorporated slowly into the mixture to obtain a viscous suspension. The latter was introduced into a mold then degassed and cured during 72h at 100°C in an oven. FTIR spectra before and after reaction are presented Figure S12. A control experiment was also performed without Lipase TL and results are presented Figure S13.a and b.

*Swelling tests.* Solubility tests were realized with 200 mg of crosslinked polymer, swollen in toluene for 1 week at 60°C. After swelling, the samples were wiped, left to equilibrate, and weighed. The solvent uptake and swelling were measured using:

$$\text{Solvent uptake [mass\%]} = \frac{W_s - W_t}{W_t} \times 100 \quad (1)$$

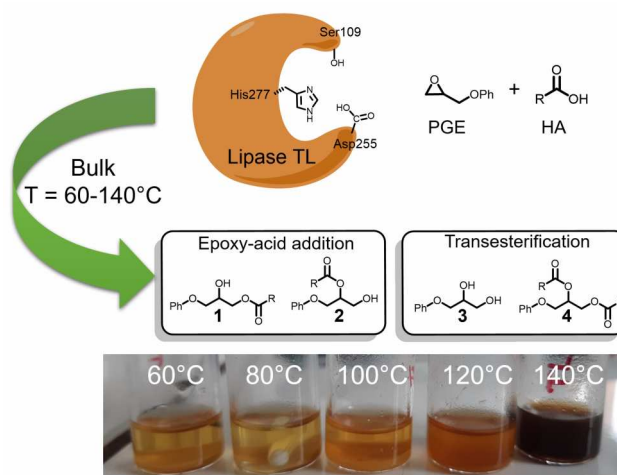
Where  $W_t$  and  $W_s$  are the weights before and after swelling, respectively. The samples were then dried under vacuum at 70°C for 48 h to measure the residue content.

*Enzyme recovery after epoxy cure.* In a 5 mL vial, Lipase TL (61.2 mg), resorcinol diglycidyl ether (1.00 g, 8.9 mmol) and hexanoic acid (1.04 g, 17.5 mmol) were successively added. After thermal treatment, *i.e.* 72h at 100°C (**RGE100**) or 12h at 120°C (**RGE120**) or 6h at 140°C (**RGE140**), the conversion was measured by  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , Figure S19) and confirmed full consumption of starting material (See Figure S19). The crude mixture was then diluted in 15 mL of THF, filtrated and wash twice with  $2 \times 15\text{mL}$  of THF. The recovered lipase was dried under vacuum at 20°C for 6h prior titration. Results obtained are presented in Figure S7 and Table S2.

## Results and discussion

Structural, analytical and functional studies on lipase from *Pseudomonas stutzeri* (lipase TL) have been already reported.<sup>34</sup> Lipase TL is a free (non-supported) enzyme and its catalytic activity is directly related to the amino acids triad side chains containing an alcohol, an imidazole and a carboxylate moiety respectively Ser-109, Asp-255 and His-277 residues; as represented schematically in Figure 1. Furthermore, lipases TL possess interesting unusual features such as the ability to recognize sterically hindered substrates (thanks to a large hydrophobic cavity) in a wide range of organic solvents (polar and non-polar).<sup>34</sup> Those

characteristics make them promising candidates for non-conventional enzymatic transformations.



**Figure 1.** Lipase TL catalyzed epoxy acid addition and transesterification reactions at different temperatures and picture of the reaction media after reaction at 60°C, 80°C, 100°C, 120°C and 140°C (from left to right)

The catalytic ability of lipase TL under epoxy curing conditions, that significantly depart from physiological ones (*i.e.* in a bulk acid/epoxy mixture at different temperatures ranging from 60°C to 140°C), was assessed by molecular model reactions (see Figure 1). 2-Phenyl glycidyl ether (PGE) and hexanoic acid (HA) were selected as model molecules as they are mutually miscible and mimic the reactive sites of classic epoxy-acid monomers. For any temperature, conversion was evaluated using the disappearance of epoxy ring signals at 3.27, 2.83 and 2.69 ppm (Figure 2). Conversions thus measured at the end of the reaction are reported in Table 1 and appear systematically higher whenever lipase TL is present. For instance, full conversion ( $\geq 99\%$ ) was observed with lipases at 100°C, 120°C, and 140°C while non-catalyzed solutions showed only 30, 32 and 37% epoxides consumption at equivalent reaction time. At lower temperature (60°C and 80°C), barely any conversion ( $\leq 5\%$ ) was recorded for the non-catalyzed reactions. Meanwhile, enzyme-catalyzed models eventually reached 50-95% conversion after 3 to 5 days under the same conditions. This compilation of results illustrates the activity of lipase TL at relatively low molar concentration and high temperatures ( $\geq 60^\circ\text{C}$ )



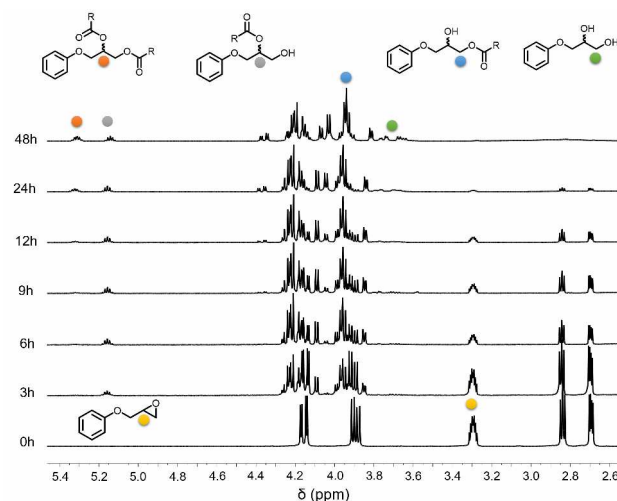
in bulk mixture. Indeed, 3.0 wt.% of lipase TL roughly corresponds to 0.0003 mol.% (with  $M = 27$  kDa, data from Meito Sangyo Ltd) as compared to epoxide functions.

**Table 1.** Lipase TL catalyzed epoxy acid addition and transesterification reactions at different temperatures (bulk,  $[PGE]_0 = [HA]_0 = 3.33$  mmol, Lipase TL = 3.0 w.%, deionized water = 0.3 w.%, temperature ranging from 60°C to 140°C) ; NC = non-catalyzed reactions, TL= Lipase TL, TDL = thermally-degraded lipase, BSA= Bovin Albumine Serum)

Label	Catalyst	Temperature (°C)	Reaction time (in h)	% conv. <sup>a</sup>	$K_{app}$ <sup>b</sup>	Trans. <sup>c</sup>
NC60	none	60	120	2	<i>n.d.</i>	Red
TL60	TL	60	120	50	0.009	Green
TL60-H <sub>2</sub> O	TL-H <sub>2</sub> O	60	120	73	0.013	Green
NC80	none	80	72	5	<i>n.d.</i>	Red
TL80	TL	80	72	88	0.030	Green
TL80-H <sub>2</sub> O	TL-H <sub>2</sub> O	80	72	95	0.048	Green
NC100	none	100	48 (72)	30 (50)	0.010	Orange
TL100	TL	100	48	98	0.115	Green
TL100-H <sub>2</sub> O	TL-H <sub>2</sub> O	100	48	≥ 99	0.199	Green
TDL100	TDL	100	48	96	0.090	Orange
BSA100	BSA	100	48	96	0.056	Orange
NC120	none	120	10	32	<i>n.d.</i>	Orange
TL120	Lipase TL	120	10	≥ 99	0.438	Orange
TL120-H <sub>2</sub> O	TL-H <sub>2</sub> O	120	10	98	0.507	Orange
NC140	none	140	4	37	<i>n.d.</i>	Orange
TL140	TL	140	4	≥ 99	1.218	Orange
TL140-H <sub>2</sub> O	TL-H <sub>2</sub> O	140	4	≥ 99	1.362	Orange

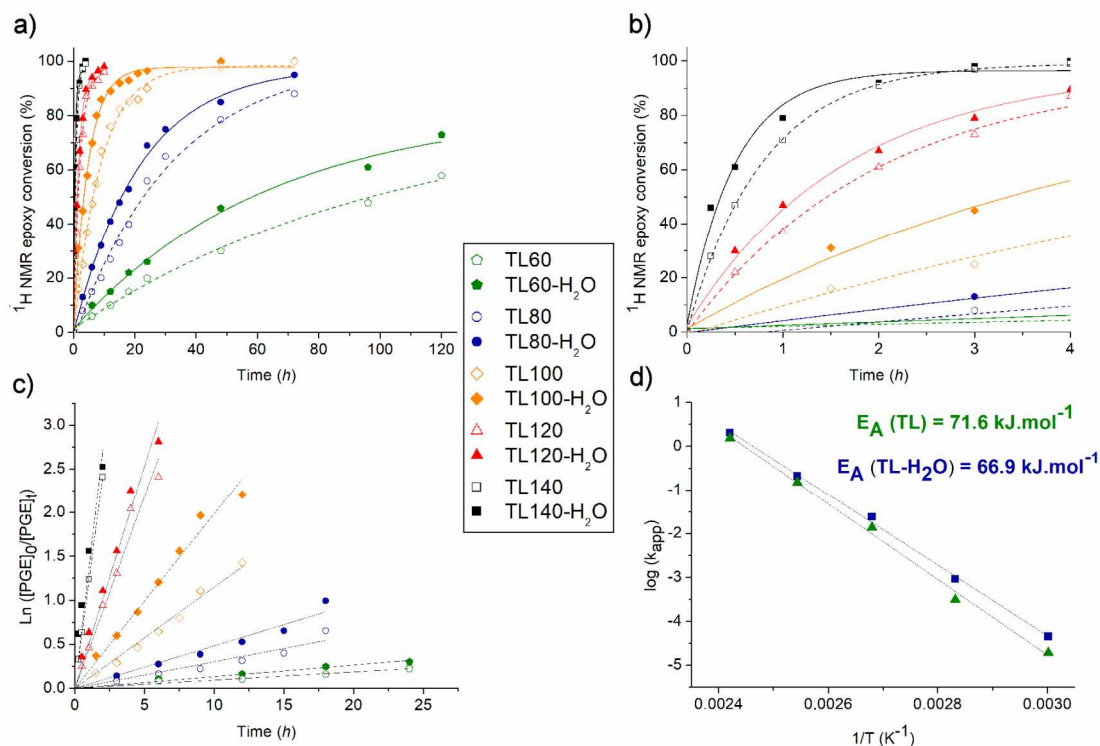
a) Conversion measured by <sup>1</sup>H NMR spectroscopy (epoxy signals) b) calculated from  $\ln([PGE]_0/[PGE]_t)$  vs. time plot presented in Figure 3. c) Transesterification evaluated by <sup>1</sup>H NMR spectroscopy: red ≤ 5 % detected transesterification products; orange ≤ 10 % detected transesterification products; green ≥ 40 % detected transesterification products.

Analysis of the crude mixtures by <sup>1</sup>H NMR spectroscopy and GC-MS (presented in Figures S1 and S2) revealed the formation of the four products shown in Figure 1: two isomers consistent with epoxy-acid additions (**1** and **2**) and two other products solely obtainable with transesterifications (**3** and **4**). In Figure 2, the NMR characteristic signals of products **1-4** are marked by color spots arising at, respectively  $\delta$  (ppm) = 4.00, 3.79-3.63, 5.31 and 5.15 ppm along with the starting material at  $\delta$  (ppm) = 3.30 ppm (epoxy signal) and allow to accurately follow the formation of the above-mentioned products at any time.

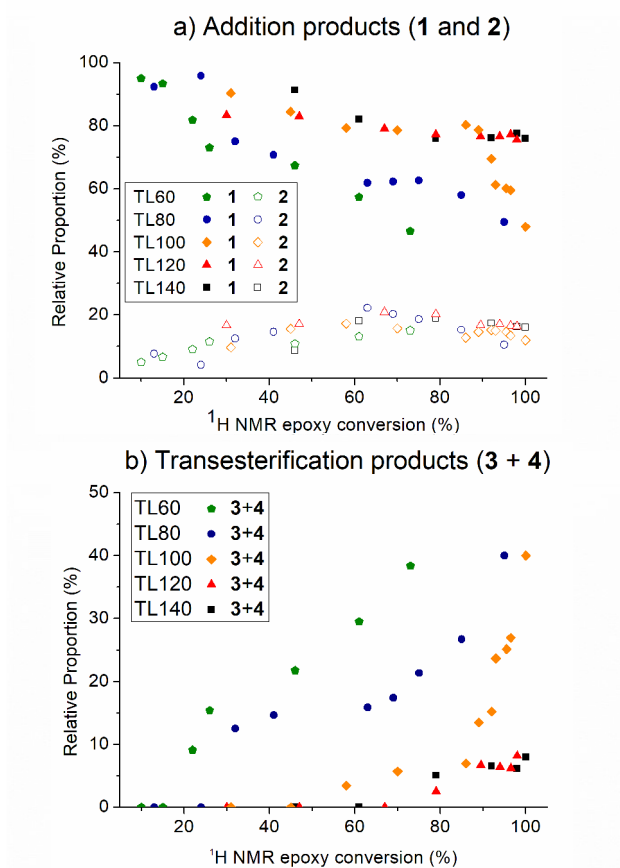


**Figure 2.**  $^1\text{H}$  NMR spectroscopy spectra ( $\text{CDCl}_3$ , 400 MHz) of the crude mixture at different time (zoom on the epoxy region 2.5-5.5 ppm) for the lipase TL catalyzed reaction at  $100^\circ\text{C}$  (TL100, LTL = 3w.%, Table 1)

By plotting the evolution of the relative proportion (*vs.* conversion) of each product at several temperatures (*i.e.* addition and transesterification reaction products), diverse selectivities and reactivities were observed (Figures 3 and 4) depending on temperature and water content. At temperatures  $\leq 100^\circ\text{C}$ , slow epoxy-acid addition and transesterification reactions occurred simultaneously, starting from 5% to 50% epoxy consumption depending on the temperature. At higher temperatures ( $120$  and  $140^\circ\text{C}$ ), only a slight trace of transesterification products (**3** and **4**) was detected despite a faster addition rate (full conversion in a few hours, Figures 3a and 3b). Interestingly, the relative proportion of product **2** (around 15%) was independent of the reaction temperature and matched the values found in controls catalyzed by 2-phenyl imidazole (2-PI, see Figure S1). The low concentration of transesterified products at high temperature, also detectable in non-catalyzed reactions (see Figure S4), suggests that the enzyme is probably denatured at  $T > 100^\circ\text{C}$  and loses its ability to catalyze transesterification reactions.



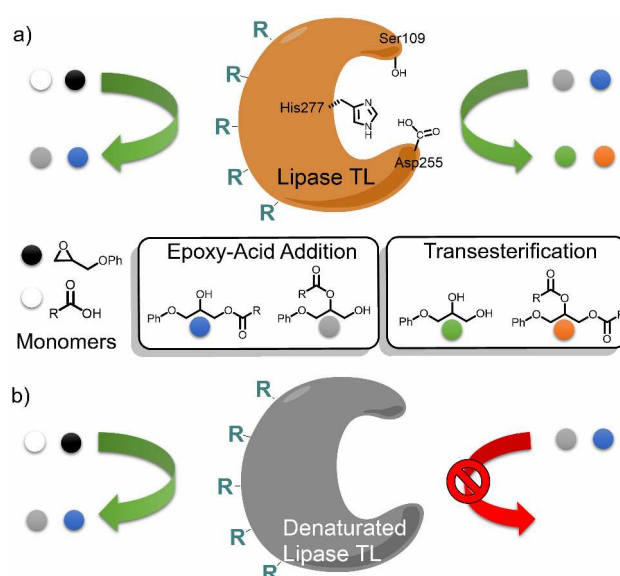
**Figure 3.** a)  $^1\text{H}$  NMR epoxy conversion as a function of time, influence of temperature and water addition b) from early stage reaction (0-4h) c)  $\text{Ln}([\text{PGE}]_0/[\text{PGE}]_t)$  vs. time: determination of  $k_{\text{app}}$  and d) Arrhenius plot for lipase-catalyzed and lipase- $\text{H}_2\text{O}$ -catalyzed addition reaction



**Figure 4.** Relative proportion vs.  $^1\text{H}$  NMR epoxy conversion of a) epoxy-acid addition product **1** and **2** and b) transesterification products (**3+4**)

Darkening of the reaction medium presented in Figure 1 also suggests denaturation of the enzyme. Surprisingly, the addition reaction was still catalyzed under those harsh conditions ( $140^\circ\text{C}$ ), meaning that the thermally-denatured enzyme was able to promote the formation of mono-esters. This unusual behavior was evidenced by employing a deliberately thermally-degraded Lipase (after a thermal treatment at  $200^\circ\text{C}$  for 3h), as a catalyst for the addition at  $100^\circ\text{C}$  (TDL-100 entry in Table 1). In this case, high conversion (96%) and similar reactivity were also measured by kinetic experiments (Figure S6).  $^1\text{H}$  NMR analysis of the crude product of reaction catalyzed by TDL (Figure S5) exhibits only signals attributed to the addition product and confirm that the thermally-denatured lipase was not able to promote transesterification. Bovine Serum Albumin (BSA) which possesses low catalytic activity towards hydrolysis (see enzymatic assays, Section 2, supporting information) was also able to catalyze the epoxy-acid addition under similar conditions. The combination of results obtained from kinetic experiments (Figure S6) with lipase TL and with denaturated-enzymes (TDL and BSA) indicates that the epoxy-acid addition is not catalyzed at the enzymes active site. It is more likely catalyzed by active R-groups such as imidazole, amines or carboxyl groups which are known to react with epoxy functionalities.<sup>29,35,36</sup> To go further and evaluate the enzymatic activity under those specific conditions, classical enzymatic assays were performed through the colorimetric titration of triolein hydrolysis. Results presented in Section 2 (ESI) clearly demonstrate the influence of the epoxy groups on the Lipase TL activity but also thermal stability. The activity measured for the neat Lipase TL (around  $500\text{ U}\cdot\text{g}^{-1}$ , Table S2 and Figure S7) was shown to increase to  $1580\text{ U}\cdot\text{g}^{-1}$  when the enzyme is mixed with 2-phenyl glycidyl ether prior titration. After thermal treatment (72h at  $100^\circ\text{C}$ ), the neat enzyme loses all ability to catalyze the hydrolysis whilst the enzyme-epoxide mixture

subjected to the same treatment still shows some activity ( $260 \text{ U}\cdot\text{g}^{-1}$ , Table S2 and Figure S7). These unexpected features prompted us to suggest a mechanism where the epoxy-acid addition is catalyzed by active side groups (**R**) for both active and denatured enzymes but transesterification only occurs when the active site is preserved or partially preserved at  $T \leq 100^\circ\text{C}$  (Figure 5.a and b). Of note, these results are in line with the good thermal stability of lipases reported by other authors (up to  $160^\circ\text{C}$  and in particular in non-aqueous media).<sup>36,37</sup>



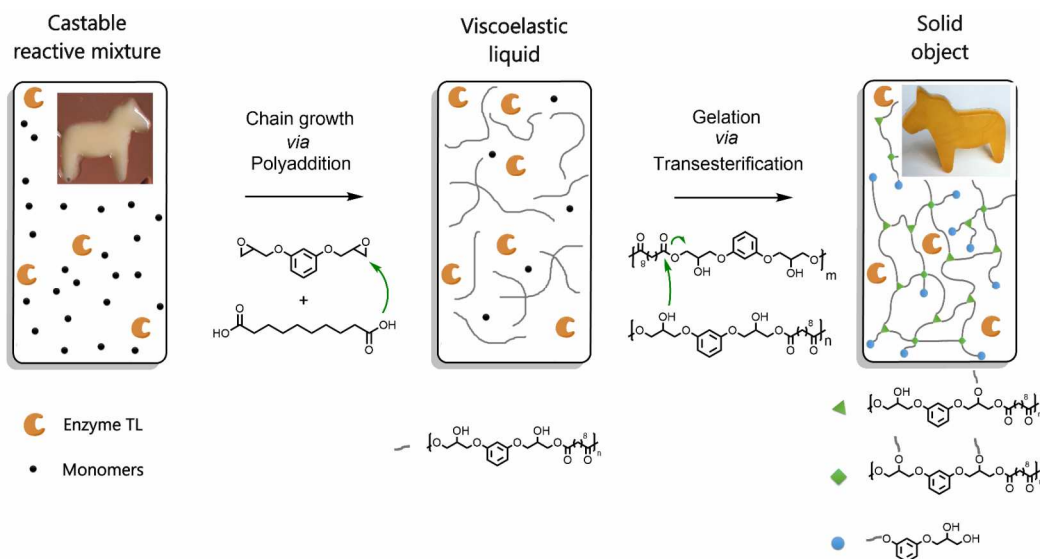
**Figure 5.** Proposed reaction pathway for a) lipase TL below  $100^\circ\text{C}$  with enzymatic active site or b) denatured lipase TL above  $100^\circ\text{C}$  (**R** = Active side groups)

Once the active site was recognized as actively playing a role in transesterification reactions, the influence of water addition on both reactions (addition and transesterification) was investigated. In non-conventional media (non-aqueous), water is commonly used to increase transesterification reactions rate for enzymatic biofuel production.<sup>38-40</sup> In these systems, water is known to play the role of lubricant and affects both enzyme activity and thermal stability (denaturation occur at lower temperature).<sup>41,42</sup> Some essential water is required to keep the enzyme active in organic media but an excess also leads to undesired side reactions (*e.g.*

hydrolysis in the case of transesterification). Therefore, an optimum water content is necessary to maintain a maximum activity and suitable stability at the same time. Kinetic experiments at different temperatures, with (0.3 wt.%) or without water, are reported in Figure 3.a and b. For the epoxy acid-addition catalyzed by lipase TL, a low amount of water (0.3 wt.%) was found to have an impact on the rate of the addition reaction. Whatever the reaction temperature (60-140°C), water accelerates the epoxy-acid addition and decreases the activation energy from  $E_{A(TL)} = 71.6 \text{ kJ.mol}^{-1}$  to  $E_{A(TL-H_2O)} = 66.9 \text{ kJ.mol}^{-1}$  (Arrhenius plot in Figure 3.c and d). The relative proportion of addition (**1** and **2**) and transesterification (**3** and **4**) products presented in Figure S9-11 demonstrate that presence of water does not change the typical temperature-dependent behavior previously described, and only increases the addition rate. Nevertheless, the addition of a larger amount of water (3 and 6 wt.%, SI Figure S9) did not accelerate the epoxy-acid addition under these conditions. Indeed, enzymatic assays (entry 9-10, Table S2) performed in epoxy-water mixture (0.3. wt.% and 6.0 wt.%) systems confirm that the addition of an excessive amount of water favors the lipase denaturation at 100°C. Of note and apart the negative impact already mentioned, *i.e.* hydrolysis side reactions and reduced thermal stability, low amounts of water are also preferable for adapting the enzymatic strategy towards bulk material synthesis. In the end, a control experiment with water (0.3 wt.%) and without lipase (Figure S6) demonstrated that water alone has no influence on conversion rates.

To assess the cure of a cross-linkable system, a prototype bio-based epoxy-acid chemical network was produced by reacting resorcinol diglycidyl ether and sebacic acid in a 1:1 [acid] / [epoxy] ratio (Figure 6). The reaction was checked using a temperature-controlled ATR-IR set-up. A cure at 100°C for 72h with Lipase TL-H<sub>2</sub>O (respectively 3 and 0.3 wt.%) was found to be the best compromise between activity (both addition and transesterification reactions occurred) and stability. The IR spectra presented in Figure S12 show complete disappearance

of acid  $\bar{\nu}_{\text{C=O}}$  and epoxy  $\delta_{\text{C-H}}$  signals at respectively  $1705\text{ cm}^{-1}$  and  $914\text{ cm}^{-1}$  along with the appearance of ester  $\bar{\nu}_{\text{C=O}}$  signal at  $1745\text{ cm}^{-1}$ . The formation of a chemically crosslinked network ( $T_g = 7\text{ }^\circ\text{C}$ , Figure S14) is further demonstrated by a swelling test (7 days in toluene at  $60^\circ\text{C}$ ) exhibiting a 50% swelling ratio and only a low amount of soluble residues ( $\leq 8.0\%$ , Figure S16). A control experiment was performed by reacting a stoichiometric amount of sebacic acid and resorcinol diglycidyl ether under the same conditions (72h,  $100^\circ\text{C}$ ) without lipase TL as curing agent. An insoluble network (solvent uptake  $\approx 45\%$ , Figure S17) with a higher soluble fraction ( $\geq 15\%$ ) is obtained. As observed in molecular studies, only incomplete conversion was achieved (NC100, 72h,  $\text{conv.} \approx 50\%$ ) and unreacted sebacic acid can be detected from FTIR analysis ( $\bar{\nu}_{\text{C=O}}$  (COOH) at  $1705\text{ cm}^{-1}$ , Figure S13.b) and DSC analysis (exothermic peak corresponding to the melting of SA, Figure S15). From kinetic experiments performed on model molecules (Figure 3 and 4), we suggest a mechanism in which simultaneous addition and transesterification, catalyzed by the enzyme, are involved in the network formation (Figure 6). Chain-growth occurs through a polyaddition mechanism between carboxylic acid and epoxy functions while gelation is obtained *via* transesterification involving  $\beta$ -hydroxy ester groups distributed along the backbone. From the product identification described in molecular model reactions, the chemical network is expected to be composed out of linear poly( $\beta$ -hydroxy esters) chains, tri- or tetra-esters crosslinks and diol-chain end moieties (Figure 6).



**Figure 6.** Reactions involved during curing the 1:1 diepoxide/di-acid liquid mixture in the presence of Lipase TL at 100°C/72h, as expected from model molecules study. Occurrence of transesterification is necessary to reach gelation from this composition.

An image of a molded elastomeric object made out of this formulation is shown in Figure 6. In order to evaluate the activity of the lipase-catalyst trapped in the epoxy matrix, dissolution tests (control network and lipase-catalyzed network) were performed in benzyl alcohol at 100 °C for 3 days and are presented Figure S18. A swollen gel was obtained in the control network prepared without any catalyst. Complete dissolution was observed for the network containing the enzyme meaning that the latter, integrated in the epoxy resin is able to perform transesterification even after curing. To go further and measure the lipase activity under different curing conditions suitable for material preparation, *i.e.* 72 h at 100 °C, 12h at 120 °C or 6h at 140 °C, a model reaction was performed between resorcinol diglycidyl ether and hexanoic acid. The choice of this monofunctional curing agent allowed to accurately reproduce the curing conditions of the epoxy resin, without inducing gelation. The enzyme was then extracted by simple precipitation/filtration in THF and its activity measured through the colorimetric titration of triolein hydrolysis. With the enzyme thus recovered, denaturation



was concluded for curing temperatures  $T > 100\text{ }^{\circ}\text{C}$  as no activity was recorded anymore for the extracted lipase subjected to curing cycles of  $120\text{ }^{\circ}\text{C}$  (12 h) and  $140^{\circ}\text{C}$  (6h) (Table S2, entry 12 and 13). However, for the cure cycle at  $100\text{ }^{\circ}\text{C}$  during 72h the extracted enzyme was still able to catalyze the hydrolysis of triolein with a measured activity of  $270 \pm 75\text{ U}\cdot\text{g}^{-1}$ , Table S2 and Figure S7). Beyond curing, the presence of an active biocatalyst integrated into the poly( $\beta$ -hydroxy esters)-based network is an attractive feature to prepare dynamic covalent networks. In general, a dynamic poly( $\beta$ -hydroxy esters) chemical network requires high temperatures to observe the exchange reactions ( $T \geq 150^{\circ}\text{C}$ ) inducing undesirable side reactions and the formation of non-exchangeable bonds.<sup>32</sup> The peculiar properties, *i.e.* transesterification exchange reactions at temperatures lower than  $100^{\circ}\text{C}$  appear as a promising strategy for the preparation of covalent adaptable networks. One of the main concerns highlighted by molecular model reactions and enzymatic assays is the denaturation of the enzyme under harsh conditions for those type of biocatalysts (epoxy-acid mixture, 72h at  $100^{\circ}\text{C}$ ). As exchange reactions (transesterification) are only catalyzed by the enzyme active site (Figure 5), denaturation induced by processing or healing might reduce the exchange efficiency after multiple cycles. The above strategy may be applied with other bio-based monomers to design networks with tunable glass transition and cross-link density, *e.g.* by using multifunctional bio-based epoxides.<sup>33,43</sup> Healable elastomers with tunable viscoelastic properties are useful in emerging technologies such as soft robotics.<sup>44</sup> Furthermore, the constant development of new bio-based epoxy monomer platforms<sup>45</sup> combined with the enzymatic approach presented in this publication offer a new route to produce more environmentally friendly materials with attractive properties.

## **Conclusion**

Molecular model reactions have demonstrated the potential of lipase TL to catalyze simultaneously epoxy-acid addition and transesterification reactions under typical epoxy cure conditions, *i.e.* in a bulk mixture of acid and epoxide and above the glass transition temperature of the target network. It was defined that the lipase active site is necessary to promote transesterification reactions and produce chemically crosslinked network from bifunctional monomers. Precise characterizations of the formed products have been then employed to determine the specific conditions (temperature and water content) required for an efficient curing process. A prototype bio-based epoxy-acyl chemical network was prepared in optimized conditions (100°C, 72h, water 0.3 wt.%), showing the potential of this enzymatic approach towards mild temperature cure of epoxy-acyl networks relevant for manufacture

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## **Supporting information**

Additional content (<sup>1</sup>H NMR analysis, GC-MS characterization, enzymatic assays and chemical network analysis) are provided in the electronic supplementary information.

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## Graphical Abstract

