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# Polylactones synthesis by enzymatic ring opening polymerization in flow

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**Abstract** Polycondensation in batch is the classical way to produce polyesters. However, this method requires harsh conditions to reach high conversions and drive the equilibrium of the reaction forward. In order to produce controlled polymers in a greener way, we worked on the enzymatic ring opening polymerization (e-ROP) of lactones using the CALB lipase immobilized on porous beads (Novozym® 435) as a catalyst and 3-phenyl-1-propanol (PP) as initiator in a flow tubular reactor made of fluorinated ethylene propylene (FEP) tubing with  $i.d = 1.55$  mm. In a short residence time of 214 s  $\epsilon$ -caprolactone (CL) was fully polymerized (conv = 100%) with a fair dispersity ( $\bar{D} = 1.30$ ). Polymerization of  $\delta$ -valerolactone (VL) (conv = 93%,  $\bar{D} = 1.27$ ) as well as copolymerization of CL and VL mixtures (conv up to 88%,  $\bar{D} = 1.24$ ) behaved similarly. By alternating the lactones, a block-polymer was obtained. As expected, primary alcohols were more efficient than secondary ones for initiating the e-ROP of lactones. Very interestingly, using cholesterol as initiator induced the formation of cyclic polylactones only. In addition, we showed the importance of the residence time as at a long time the formed polymer was depolymerized by the enzyme. We demonstrated also that the enzyme may be reused more than ten times with maintaining its high efficiency. The whole study shows that the use of tubular reactor for performing e-ROP improves its control as shown by the low  $\bar{D}$  values and decreases the residence time needed to reach high conversions compared to similar reactions in batch.

## Key Words

Enzymatic Ring Opening Polymerization (e-ROP), Flow system, Polylactones, Novozym® 435

## **I Introduction**

The importance of polyesters comes from their wide uses in our daily life. Polyesters are non-toxic and biocompatible which explains their use in both medical and pharmaceutical fields [1]. Ring Opening Polymerization (ROP) is mainly used to produce polyesters from the corresponding cyclic monomers [2]. Many metal based catalysts are used in polyesters synthesis: Aluminium [3, 4], Zinc [5, 6] and Tin [7, 8]. Among these catalysts, Tin(II)octanoate is the most commonly used. Poly ( $\epsilon$ -caprolactone) (PCL) was successfully synthesized in flow using FTEP tubes with metal based catalyst  $\text{Sn}(\text{OTf})_2$ . The reaction time was enough to reach high conversions and good molecular weight dispersity of polymers under controlled reactions [9]. In addition, they used an initiator which contains hydroxyl and thiol functions. It conserved the thiol-end fidelity after the chemoselective polymerization towards alcohol [10]. However, metal based catalysts are not safe in catalyzing the synthesis of biodegradable polyesters destined for biomedical use [1, 11].

Green chemistry has now become a very attractive field. It represents a new point of view in chemistry, promoting the use of green starting materials, solvents and process that respect the environment and afford products which are not damaging our environment [12]. In this paper, we focused on green synthesis of macromolecules. Enzymes; performing clean reactions with no side products and providing high selectivity with respect to enantio-, regio- and chemo-selectivities are the archetype of green catalysts. No conventional catalytic system affords such a degree of selectivity. In addition, enzymes are natural, renewable and nontoxic. Furthermore, enzymes are recyclable which allows their reuse multiple times [13-16]. The traceless green solvent, supercritical carbon dioxide ( $\text{scCO}_2$ ) can be used for promoting enzymatic polymerization [17]. Lipases are a class of enzymes which hydrolyse fatty acid esters and they are the most commonly used in enzymatic polymerization [15, 16]. Novozym 435® from Novochem is a lipase B (CALB) immobilized on an acrylic resin which is commercially available [18]. Novozym 435® has been found the most effective enzyme to promote the ring opening polymerization of lactones [19].

Flow microreactors allow high performance organic syntheses. High surface to volume ratio of microreactors, short diffusion paths and more efficient heat and mass transfer compared with conventional platforms explain these improved performances [20-23]. The reaction parameters are easily controlled. For example, the reaction time is adjusted by the simple variation of the flow rate or of the microchannel length. Polymer synthesis has also been developed in continuous flow microreactors [24, 25]. In a seminal work, Gross and Beers employed immobilized enzyme in continuous flow system to achieve  $\epsilon$ -caprolactone (CL) ring opening polymerization in toluene [26]. The polymerization took place in an Aluminium microchannel reactor coated with Kapton® film. In comparison with batch system, they obtained a higher number-average molecular weight ( $M_n$ ) of PCL in less reaction time proving the advantages of using N435® beads in a microreactor to catalyze the ROP of CL. Neither conversion nor chemoselectivity are affected when using toluene saturated with water [27]. These works have paved the way to several polylactones synthesis in micro-flow system [28-30]. In addition, Zhu et al. examined the chemoselectivity towards hydroxyl versus thiol functions via enzymatic ring opening polymerization of  $\delta$ -valerolactone (VL) in flow mode as well as its copolymerization where they obtained interesting results compared to the batch [31].

In order to develop a green polyester synthesis, we combined the advantages of micro-flow system, ROP and green catalyst using a homemade setup easily built in any laboratory. CL and VL polylactones were synthesized as well as their copolymers via enzymatic ring opening. A comprehensive study of the parameters (initiators, flow rate, temperature and reaction time) of each reaction was performed. In this study, we were able to show the special behavior of cholesterol affording cyclic polyesters. The formed polymers were characterized by  $^1\text{H}$  nuclear magnetic resonance spectroscopy (NMR), mass spectrometry (MS) and gel permeation chromatography (GPC). Furthermore we studied the degradation of the formed polyesters to understand the kinetics and therefore to optimize the eROP. Our results show that the combination of enzymatic ring opening polymerization and micro-flow technology open the access to well-defined and cost effective polymer synthesis.

## **II Methods and materials**

### *II.1 Chemicals*

Initiators (1-propanol, 1-octanol, 3-methyl-1-butanol, 3-phenyl-1-propanol (PP), 4-phenyl-2-butanol, 1-pyrenebutanol and cholesterol), solvent (anhydrous toluene), ionization agent for MALDI analysis sodium iodide (NaI) and matrix dihydroxybenzoic acid (DHB) were purchased from Sigma-Aldrich® (St. Louis, MO, USA) and used as received. The two monomers:  $\delta$ -valerolactone (VL) and  $\epsilon$ -caprolactone (CL) were purchased from TCI (Tokyo Chemical Industry, Tokyo, Japan) and conserved on activated molecular sieves. Novozym 435® (N435®) beads was purchased from STREM chemicals (Newburyport, MA, USA).

### *II.2 Analytical methods*

Proton nuclear magnetic resonance spectra ( $^1\text{H}$  NMR) were recorded on an AVANCE 300 spectrometer (300 MHz) (Bruker Biospin, Wissembourg, France) using tetramethylsilane (TMS) as the internal standard and using  $\text{CDCl}_3$  as solvent. The peak of non-deuterated chloroform resonates at 7.23 ppm. The experiments were carried out at 293 K.

Size exclusion chromatography (SEC) was performed on a Prominence chromatography chain (Shimadzu, Kyoto, Japan) fitted with a Refractive Index (RI) detector (RID-20A) and an UV detector (SPD-20A). The columns (KF-802 and KF-803L from Shodex (Tokyo, Japan)) were eluted with tetrahydrofuran (THF) at a flow rate of 1 mL/min at 30 °C. The samples were previously prepared by dissolving 10 mg of sample in 1 mL THF. The solution was then filtered through a PTFE filter with a pore diameter of 0.45  $\mu\text{m}$ . A volume of 20  $\mu\text{L}$  was injected into the size exclusion chromatography to carry out the analysis. The SEC was calibrated with poly (styrene) standards. The number average molecular weight was determined from the UV detector absorbance.

Matrix assisted laser desorption ionization-time of flight mass spectra (MALDI-TOF MS) were recorded at 20 kV in positive ion mode on an ABISciex (Framingham, MA, USA) 4800 plus mass spectrometer. Samples, NaI were dissolved in THF at a concentration of 5  $\text{mg}\cdot\text{mL}^{-1}$  and dithranol matrix at 15  $\text{mg}\cdot\text{mL}^{-1}$ . These three solutions were mixed in a

volume ratio of 1:1:1. Then, 0.1  $\mu$ L of the final solution was spotted on the target plate. The mass range was set to  $m/z$  200–5000. The applied laser's power was 4500 (arbitrary value) and 1000 laser shots were accumulated for each scan. High resolution MALDI mass spectra were acquired on a Solarix XR FTICR instrument fitted with a 9.4 T actively shielded superconducting magnet and a dynamically harmonized cell (Bruker Daltonics, Bremen, Germany). This instrument was equipped with both laser desorption ionization source (Smartbeam® II, Nd:YAG harmonic 3 laser at 355 nm) and an electrospray source. Matrix-assisted laser desorption/ionization Fourier transform ion cyclotron resonance (MALDI-FT-ICR) mass spectra were acquired in positive ion mode. The mass range was set to  $m/z$  200–5000. The applied laser power was 20% and 500 laser shots were accumulated for each scan. Samples were deposited on the plate as previously described.

### *II.3 Enzymatic ring opening polymerization (e-ROP) of lactones in FEP microreactor*

The enzyme immobilized tubular reactor was made from fluorinated ethylene propylene (FEP) tubing (i.d. 1.55 mm, length 25 or 50 cm, Cluzeau Info Labo (C.I.L.), Sainte-Foy-La-Grande, France) fitted with 8-mL stainless-steel syringe (thread 1/4–28 inch,  $\varnothing = 1/16$  inch, PC5 702268, Harvard Apparatus) powered by a Harvard Apparatus (Holliston, MA, USA) PHD ULTRA XF syringe pump as shown in Fig. S1. The tube outlet was plugged by cotton to hold the enzymes beads after what the tube was filled with N435® beads. The enzyme bead load was 100 mg (10%w of CL) in the 25 cm long of FEP tube in which we performed the e-ROP of CL and 200 mg (20%w of VL) in the 50 cm one for the VL e-ROP. The volume of the tubular reactor was estimated by flowing toluene through the reactor and estimated to be 0.28 and 0.55 mL, respectively for the 25 and 50 cm reactors. The stock solution lactone and initiator in dried toluene was prepared using Schlenk technique under nitrogen. All reactions were carried out following the same equivalents: 20/1/40 respectively for the monomer, the initiator and toluene (solvent). The mixture was transferred into the 8 mL stainless steel syringe under nitrogen atmosphere. The enzyme immobilized tubular reactor was flushed with dried toluene to remove the moisture and air. The stock solution was pumped into the tubular reactor at the desired flow rate, and the reactor was placed into a water bath at

the specific temperature. The residence time was calculated from the flow rate and the residence volume of the tubular reactor filled by N435® beads (Eq. S1). The products were collected after precipitation in the cold methanol, filtration, and drying (see SI). We verified that no polymerization was taking place in absence of the enzyme N435® beads neither at room temperature nor at 70 °C (Table S3, runs 6 and 7), proving that the polymerization is immediately quenched once the reaction medium is no longer in contact with the enzyme.

#### *II.4 Enzymatic ring opening copolymerization (e-ROCP) of lactones in the integrated FEP microreactor system by sequential addition.*

The enzyme immobilized tubular reactor for copolymerization was an extension of the previous one. The end of the first FEP tube (diameter = 1.55 mm, length = 25 cm) was connected to a tee mixer fed by a second 8 mL stainless steel syringe powered by the same syringe pump, and connected to a FEP tube (diameter = 1.55 mm, length = 50 cm). The stock solution (A) contains the first monomer (CL), the initiator and toluene as solvent and the stock solution (B) contains the second monomer (VL) and toluene as solvent. A and B stock solutions were pumped into the tubular reactor at the same flow rate (0.067 ml.min<sup>-1</sup>). After solutions (A) and (B) had been mixed, the flow rate in the second reactor became double of that in the first one (0.134 ml.min<sup>-1</sup>), so that residence times in the first and the second tube are the same (4 min). The reactors were placed into water bath at 70 °C. The products were collected after precipitation in the cold methanol, filtration, and drying as described in SI.

#### *II.5 Degradation of polylactones by the Novozym 435®*

In order to verify the degradation of polylactones by the action of the enzyme, we prepared PCL and PVL. A specific mass of each polymer in toluene (5 ml) was injected in a FEP tubular reactor containing 300 mg of N435® beads. Three stages were studied: (i) stage 1- the initial polylactone in its dried form; (ii) stage 2- the solution recovered after passing the initial polylactone through the tubular reactor filled of N435®

beads, so before its purification and (iii) stage 3- the final product recovered after purification of the later solution (precipitation, filtration and drying).

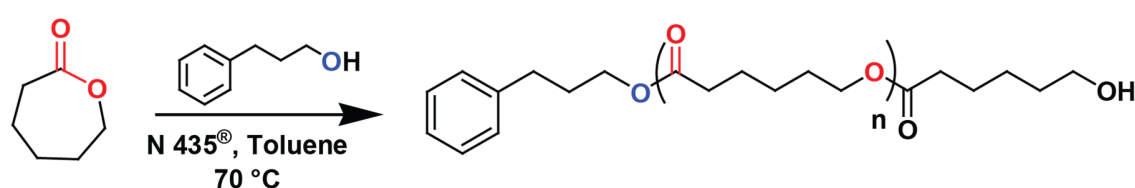
The polymers were characterized by Matrix-assisted laser desorption/ionization Fourier transform ion cyclotron resonance mass spectrometry (MALDI-FTICR-MS) at each stage to detect possible variations in their molecular weight dispersity as well as their chains structure.

### III Results and discussion

#### *III.1 Enzymatic ring opening polymerization (e-ROP) of lactones in FEP microreactor.*

##### *III.1.1 Poly ( $\epsilon$ -caprolactone) synthesis.*

Following Gross et al.'s pioneering work [26, 27], we studied CL conversion versus residence time by e-ROP (Scheme 1) in flow in dry toluene using immobilized Novozym N435 (100 mg) and 3-phenyl-1-propanol as initiator with a monomer to initiator concentration ratio ( $[M]/[I]$ ) of 20 at a concentration of 4.5 M. The microreactor used in this study had approximately the same cross-section as the one used by Kundu et al. [26, 27] (1.9 mm<sup>2</sup> versus 2.0 mm<sup>2</sup>) and the same length (250 mm versus 260 mm). In a recent study Zhu et al [29] used a larger cross section of 11.34 mm<sup>2</sup> and a slightly longer length (300 mm).



**Scheme 1.** e-ROP of CL.

Table 1 shows that at room temperature (20 °C), the CL conversion increased from 15% to 94% when the residence time was increased from 0.5 min to 10 min, and above 10 min the conversion remains at 94% (Fig. 1). The polymerization followed a first-order kinetics during the whole course of the reaction as the logarithm of the monomer concentration vs. residence time (min) exhibited a linear relationship. The slope gave a



rate constant of  $0.004 \text{ s}^{-1}$  with a coefficient of correlation  $R^2$  of 0.98 (Fig. 2). This linear correlation indicates a good control on the polymerization. During the propagation step the concentration of active centers was not altered and no termination reactions occurred [32]. In agreement with this kinetics, GPC showed a pretty narrow molecular weight dispersity ( $\bar{D} = 1.35$ ). The MALDI-TOF spectrum (Fig. 3) presented a bell-shaped peak distribution in agreement with GPC. A negligible presence of macrocycles was also detected. We repeated the same study but at  $70^\circ\text{C}$ . As indicated in Table S1, the conversion increased from 30% to 100% when the residence time was increased from 15 s to 240 s, and above 240 s the conversion remains at 100% (Fig. S4). So, at  $70^\circ\text{C}$  a lower residence time was enough to afford complete conversion. From the linear kinetic plot presented in Fig. S5 a rate constant of  $0.011 \text{ s}^{-1}$  was obtained with a coefficient of correlation  $R^2$  of 0.96. This first order kinetic rate constant is in agreement with Kundu et al [26] who also found a rate constant of  $0.011 \text{ s}^{-1}$  for the same reaction. GPC analysis showed that the molecular weight dispersity is slightly better at  $70^\circ\text{C}$  than at  $20^\circ\text{C}$  (respectively  $\bar{D} = 1.30$  and  $\bar{D} = 1.35$ ). This molecular weight dispersity is better than those Kundu et al [26] who showed the presence of oligomers and slightly higher than the molecular-weight dispersity reported by Zhu et al [29] in approximately the same conditions ( $\bar{D} = 1.12$  and  $1.17$  for  $([M]/[I])$  of 10 and 30). In all cases flow mode provided lower molecular weight dispersity than batch since under the same conditions Zhu et al [29] obtained PCL of  $\bar{D} = 1.48$  with a much higher reaction time of 14 h. As shown below the molecular-weight dispersity is sensitive to the nature of the initiator (see Table 3). The use of 6-mercapto-1-hexanol as initiator may explain their lower molecular-weight dispersity. In the MALDI-TOF spectrum (Fig. S6) two other minor species of PCLs were detected, chains initiated by  $\text{H}_2\text{O}$  and macrocycles which result from their cyclisation, but the overwhelming majority remains PP end chains (Fig. 4).

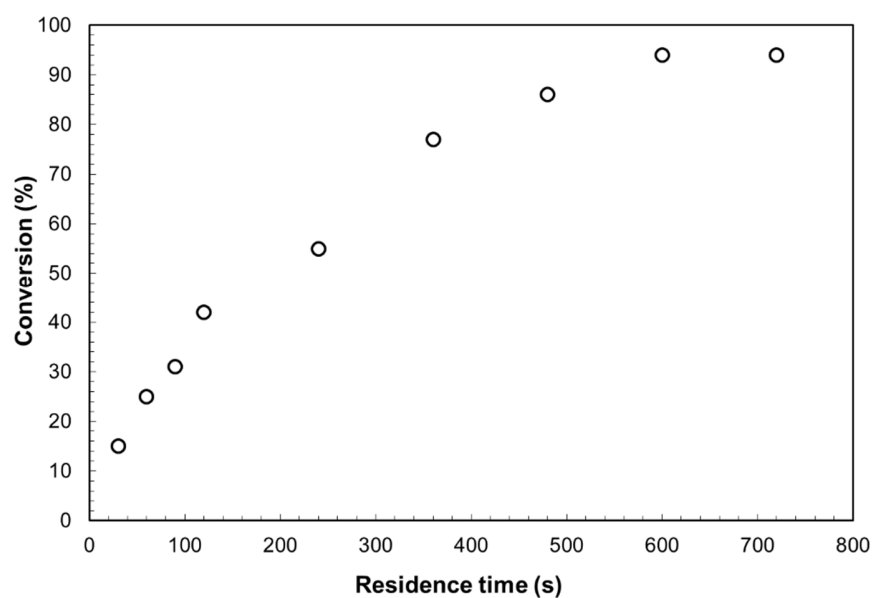
**Table 1**

PCL produced by e-ROP in flow at room temperature.<sup>a</sup>

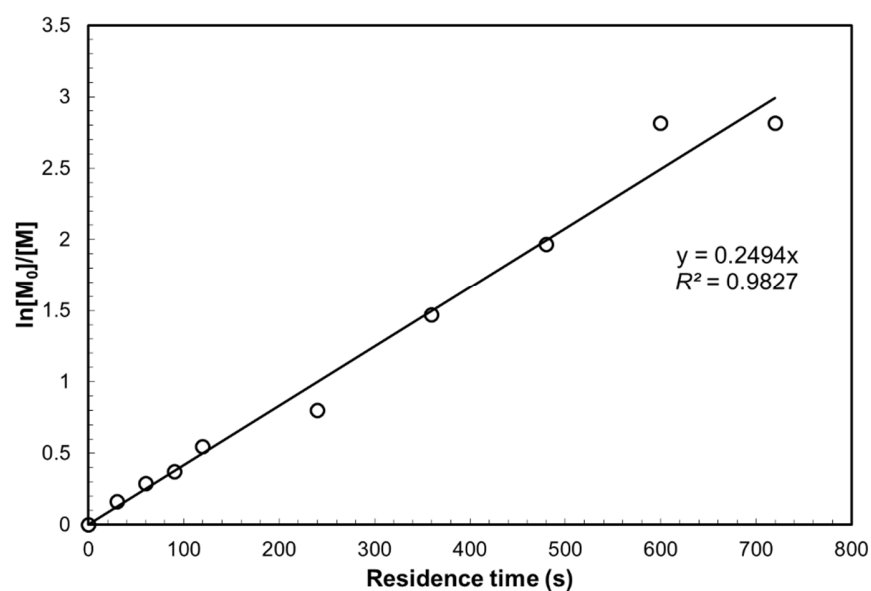
<i>Residence time</i> (min)	0.5	1.0	1.5	2.0	4.0	6.0	8.0	10	12
Conv. (%) <sup>b</sup>	15	25	31	42	55	77	86	94	94
$M_{n,NMR} \times 10^{-3}$ (g/mol) <sup>a</sup>	-	-	-	1.9	3.0	2.1	1.9	2.3	2.1

240 <sup>a</sup> [CL] = 4.5 M and [PP] = 0.225 M in toluene

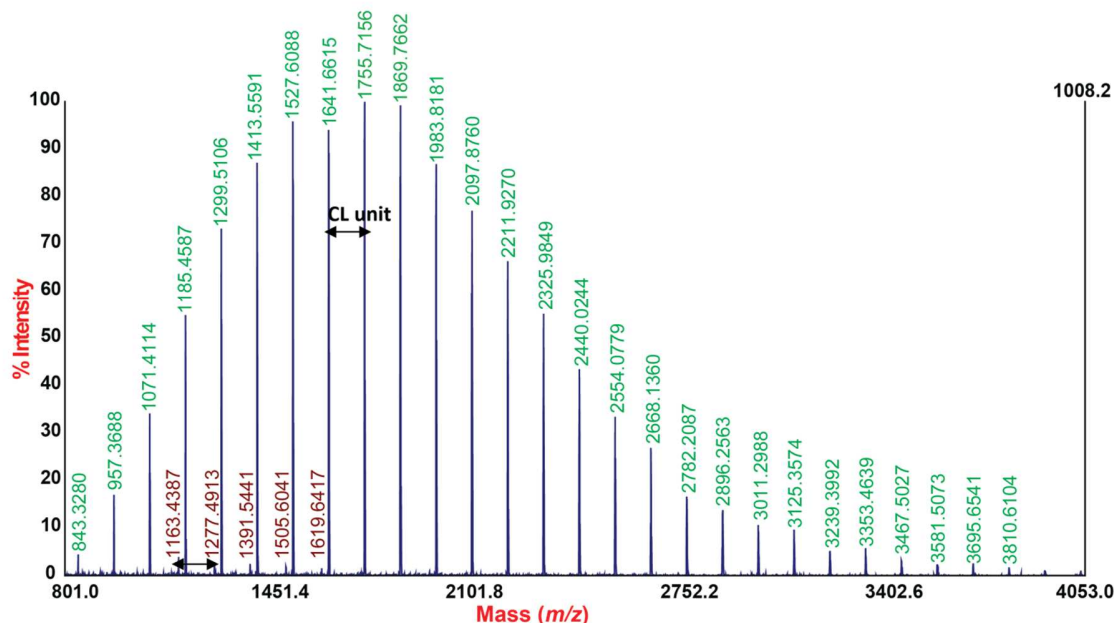
241 <sup>b</sup> Determined by <sup>1</sup>H NMR.



242  
243 **Fig. 1.** Conversion (%) of PCL versus residence time (s) at room temperature.  
244



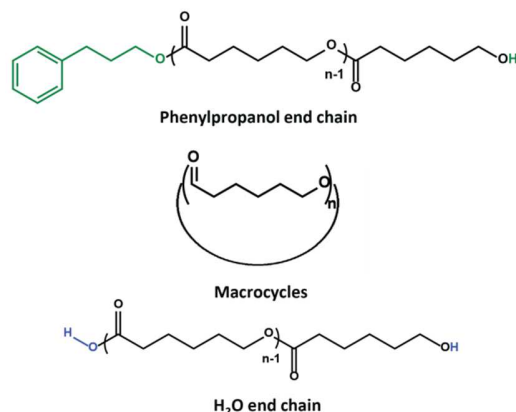
245  
246 **Fig. 2.** Semilogarithmic kinetic plot for e-ROP of CL at room temperature in flow.  
247



**Fig. 3.** MALDI-TOF MS of PCL initiated by PP in flow at room temperature using a residence time of 600 s.

Green, chains initiated by PP:  $M = (M_{\text{monomer}} \times \text{nb monomer}) + M_{\text{PP}} + M_{\text{Na}^+}$

Red, macrocycles:  $M = (M_{\text{monomer}} \times \text{nb monomer}) + M_{\text{Na}^+}$

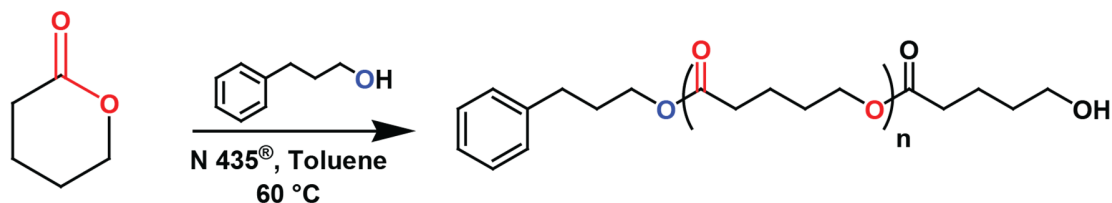


**Fig. 4.** PCL with different end chains.

### III.1.2 Poly( $\delta$ -valerolactone) synthesis.

The same study previously described with CL was repeated on VL at 60 °C (Scheme 2) also in dried toluene and using PP as initiator with a  $[M]/[I]$  of 20. VL is a more difficultly

polymerized compared to CL [33, 34], therefore we increased the amount of N435® beads from 100 mg to 200 mg.



Scheme 2. e-ROP of VL.

Table S2 shows that at 60 °C, the conversion of VL increased from 12% to 93% when the residence time was increased from 20 s to 214 s (Fig. S7). The linear relationship between the logarithm of the monomer concentration vs. residence time (s) indicates that the polymerization followed a first order kinetics. The rate constant and the coefficient of correlation  $R^2$  given by the slope are 0.012 s<sup>-1</sup> and 0.96 respectively (Fig. S8). This rate constant, which is almost equivalent to the one for CL at 70 °C, was obtained by doubling the quantity of N435® beads. This result is in agreement with van der Mee et al. [34] who gave a Michaelis-Menten relationship in which the Michaelis constant  $K_M$  is almost the same for CL and VL but the catalytic rate constant  $k_{cat}$  is nearly the double for CL, leading to polymerization rates for CL that are twice as high. This linear relationship proved again the good control of the polymerization progress, also verified by GPC, which gave a good narrow molecular weight dispersity ( $\bar{D}$  = 1.27). This value is slightly higher than those obtained by Zhu et al [31] in almost comparable reactions conditions ( $\bar{D}$  = 1.13 and 1.16 for [M]/[I] of 10 and 30) using a larger cross section tube compared to us (11.34 mm<sup>2</sup> versus 1.9 mm<sup>2</sup> respectively) with a shorter length (300 mm versus 500 mm). However, a much higher residence time of 20 min was required to reach the highest conversions of 98%. In contrast, a residence time of 214 s ( $\approx$  3.5 min) was enough in our case at [M]/[I] = 20 to reach the highest conversion of 93%. The same reaction performed in batch system gave widely dispersed polymers ( $\bar{D}$  = 1.35 for [M]/[I] of 30) for a higher reaction time of 600 min. The MALDI-TOF spectrum (Fig. S9) revealed the presence of two other families of species, chains initiated by H<sub>2</sub>O and macrocycles, although the dominant one still being the PP end

chains. Above 214 s of residence time the molecular weight decreased, which can be explained by the beginning of the depolymerization of the formed polymer [35] by the enzyme that will be discussed later in section IV. Moreover, we carried out the e-ROP of VL at room temperature (Table 2). Within 600 s (10 min), we were able to reach the complete conversion of 100% (Table 2, Run 2) with approximately the same dispersity as the one recorded at 60 °C ( $\bar{D} = 1.28$  vs  $\bar{D} = 1.27$ ). Remarkably, the conversion and dispersity obtained were the same at room temperature as at 60 °C. This residence time remains lower than those published in the work of Zhu et al [31] at 60 °C in the conditions cited previously (10 min vs 12 min and 20 min). Interestingly, the MALDI-TOF spectrum indicates that the two minor species are almost suppressed at room temperature (Fig. 6).

**Table 2**

PVL produced by e-ROP in flow at room temperature.<sup>a</sup>

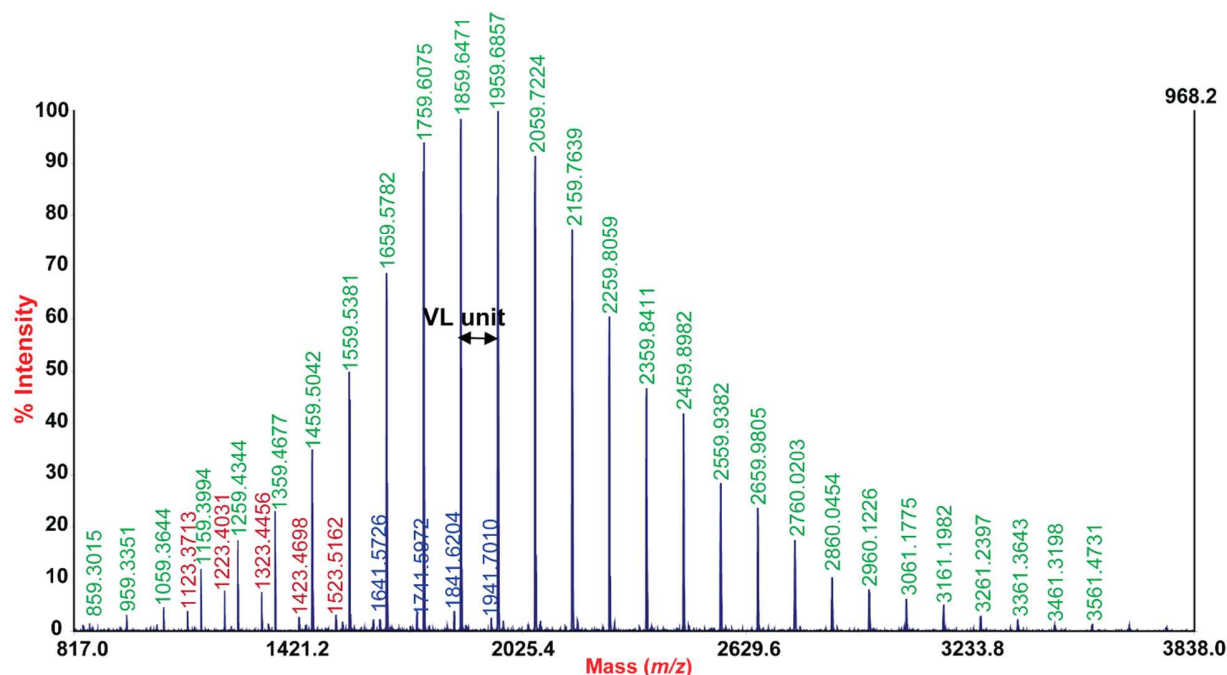
Run	Residence time (s)	$M_{n,theo} \times 10^{-3}$ (g/mol) <sup>b</sup>	$M_{n,NMR} \times 10^{-3}$ (g/mol) <sup>c</sup>	$\bar{D}^d$	Conv (%) <sup>c</sup>
1	480	1.9	2.5	-	97
2	600	2.3	2.0	1.28	100

<sup>a</sup> [VL] = 4.5 M, [PP] = 0.225 M in toluene using 200 mg of N435® beads

<sup>b</sup> Calculated from  $([M]/[I])Conv. \times M_w(VL) + M_w(PP)$ .

<sup>c</sup> Determined by <sup>1</sup>H NMR.

<sup>d</sup> Determined by GPC.



**Fig. 5.** MALDI-TOF of PVL e-ROP initiated by PP in flow at room temperature using a residence time of 600 s.

Green, chains initiated by PP:  $M = (M_{\text{monomer}} \times \text{nb monomer}) + M_{\text{PP}} + M_{\text{Na}^+}$

Red, macrocycles:  $M = (M_{\text{monomer}} \times \text{nb monomer}) + M_{\text{Na}^+}$

Blue, chains initiated by residual water:  $M = (M_{\text{monomer}} \times \text{nb monomer}) + M_{\text{water}} + M_{\text{Na}^+}$ .

### III.2 Effect of initiators on enzymatic ring opening polymerization (e-ROP).

As shown by Table 3, we used different alcohols as initiator to polymerize CL and VL to study their effects on polymers molecular weight and molecular weight dispersity. We started by primary alcohols which differ in their carbon chain length and in their steric hindrance. Propanol gave a PCL of  $\bar{D} = 1.24$ , elongating the chain carbon length from C3 to C8 using octanol increased this value to  $\bar{D} = 1.28$ . MALDI-TOF spectra show a bell-shaped peak distribution for both propanol (Fig. S10) and octanol (Fig. S11) initiators in which macrocycles and H<sub>2</sub>O end chains are in minority in both cases. 3-methyl-1-butanol, a primary branched alcohol, increased the molecular weight dispersity compared to the two previous linear alcohols ( $\bar{D} = 1.24$  and  $1.28$  vs  $\bar{D} = 1.4$ ) and afforded a greater quantity of macrocycles (Fig. S12). Pyrenebutanol, a primary but bulky alcohol, gave the highest molecular weight dispersities among primary alcohols both in CL polymerization ( $\bar{D} = 1.44$ ) and VL polymerization ( $\bar{D} = 1.39$ ). MALDI-TOF

spectra present two major species, chains with initiator extremity and macrocycles (Figs S13 to S14), the former being much more abundant. When using 4-phenyl-2-butanol (a secondary alcohol) structurally close to 3-phenyl-1-propanol, the MALDI-TOF spectrum revealed the presence of same species macrocycles and chains initiated by the initiator (Fig. 6) as for 3-phenyl-1-propanol but the quantity of macrocycles increased. When cholesterol (a bulky secondary alcohol) was taken as initiator, polymers with the appearance of cotton were obtained (Fig S15) instead of powder like for the other synthesized polyesters. MALDI-TOF spectrum only showed the formation of macrocycles (Fig. 7). The GPC analysis, calibrated with polystyrene, gave a molecular weight for this polymer  $M_n = 19 \times 10^3$  g/mol and a molecular weight dispersity  $\mathcal{D} = 1.6$  (Table 3). However, cyclic polymers exhibit higher molecular weight by GPC, leading to the conclusion that these values are overestimated [36]. The appearance of cotton for this polymer is agreement with the unique properties of cyclic polyesters leading to specific applications in biology in particular [37, 38]. Cholesterol behaved similarly with VL where macrocycles formation was obtained in addition to H<sub>2</sub>O end chains (Fig. S16). So as expected, primary alcohols were more efficient at initiating the e-ROP of lactones than the secondary ones, as the propagating chain implies a primary alcohol [39]. The lower activity of secondary alcohols has two consequences; (i) the high percentage of initiation by residual water and (ii) the more important formation of macrocycles as the acid extremity is not esterified. As an illustration 4-phenyl-2-butanol gave a high percentage of macrocycles (Fig. 6) contrary to 3-phenyl-1-propanol (PP) (Fig. 6). It must be pointed out that water cannot be completely removed as the CALB requires a minimum of free water to be active [40]. Reversible conformational changes of CALB occurred upon interaction with substrates. The presence of free water on the surface of CALB maintains its flexibility during these changes by acting as lubricant [41]. Therefore, no attempt was made to work in a totally anhydrous toluene.

**Table 3**

e-ROP of CL<sup>a</sup> and VL<sup>b</sup> using different alcohols as initiators in flow<sup>c</sup>.

Initiator	Monomer	$M_{n,GPC} \times 10^{-3}$ (g/mol) <sup>d</sup>	$M_{w,GPC} \times 10^{-3}$ (g/mol) <sup>d</sup>	$\bar{D}^d$	Conv (%) <sup>e</sup>
1-propanol	CL	5.1	6.3	1.24	88
1-octanol	CL	5.2	6.5	1.28	95
3-methyl-1-butanol	CL	3.7	5.2	1.40	-
3-phenyl-1-propanol	CL	5.1	6.6	1.30	95
3-phenyl-1-propanol <sup>f</sup>	CL	3.4	4.6	1.35	94
3-phenyl-1-propanol	VL	4.1	5.2	1.27	93
3-phenyl-1-propanol <sup>f</sup>	VL	3.7	4.8	1.29	100
Pyrenebutanol	CL	3.3	4.8	1.44	96
Pyrenebutanol	VL	3.7	3.8	1.39	90
4-phenyl-2-butanol	CL	5.7	7.0	1.24	90
Cholesterol	CL	19	23.4	1.23	91
Cholesterol	VL	6.3	10.1	1.60	78

<sup>a</sup> Reaction temperature is 70 °C, in toluene, [M]/[I](mol) = 20, 100 mg of N435.

<sup>b</sup> Reaction temperature is 60 °C, in toluene, [M]/[I](mol) = 20, 200 mg of N435.

<sup>c</sup> Reactions in flow system with residence time of 240 s. [M] = 4.5 M, [I] = 0.225 M.

<sup>d</sup> Determined by GPC.

<sup>e</sup> Determined by <sup>1</sup>H NMR.

<sup>f</sup> Reactions at room temperature.





Red, macrocycles:  $M = (M_{monomer} \times nb\ monomer) + M_{Na^+}$



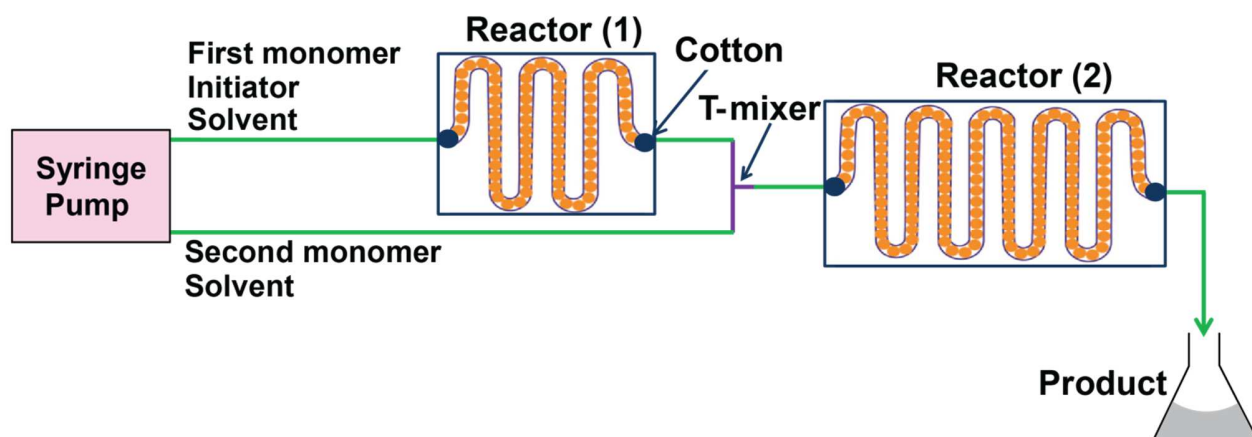
Blue, chains initiated by residual water:  $M = (M_{monomer} \times nb\ monomer) + M_{water} + M_{Na^+}$

In view of the results obtained with cholesterol, we carried out the e-ROP of CL at 70°C in the absence of added initiator. As shown in Table S3, we obtained 100% of conversion using 100 mg of N435® beads at a residence time of 120 s or 240 s (S3, entries 1 and 2). In addition, 44% of conversion was obtained at room temperature in 240 s of residence time (Run 3). Increasing the quantity of N435® beads to 200 mg at a residence time of 240 s gave the same results showing that the obtained polymer is resistant to depolymerization (Run 4). Unsurprisingly the polymers had the appearance of cotton exactly like those obtained when using cholesterol as an initiator. MALDI-TOF spectra also indicate the presence of macrocycles only (for an example of MALDI-TOF spectrum see Fig. S17). Nevertheless, the GPC of these polymers showed a much wider molecular weight dispersity [ $D = 1.99-2.68$ ]. Epoxidized cholesterol is specifically acetylated in presence of vinylacetate by N435® beads, showing that cholesterol is a substrate for immobilized CALB [42]. In other words, even if it not directly implied in lactone polymerization, cholesterol modulates the CALB activity. The maximum CALB which can be immobilized on Lewatit VP OC 1600 support is 30 mg per gram [43]. As the molecular weight of CALB is around 33 kDa the quantity of CALB enzyme in the reactor which contains 100 mg or 200 mg of N435® beads was around 100 pmol for CL polymerization and 200 fmol for VL polymerization. The quantity of cholesterol in the reactor was 63  $\mu$ M and 126  $\mu$ M for CL and VL respectively. Hence, cholesterol is present in a more than 100-fold excess compared to CALB. CALB has a stereospecific pocket for secondary alcohols [44] and the non-reacting enantiomer during kinetic resolution modulates its reactivity [45], which may explain the effect of a large excess of cholesterol. Another explanation is the complexation of cholesterol to the surface of the Lewatit VP OC 1600 support and the modification of the hydrophobicity of the enzyme environment, which also modulates its reactivity [43].

### *III.3 Enzymatic ring opening copolymerization (e-ROCP) of lactones to produce di-block copolymer in the integrated microreactor system by sequential addition*

We performed the copolymerization of CL and VL at 70 °C (Table 4) by sequential addition using the integrated microreactors with CL fed in the first reactor and CL after polymerization and fresh VL in the second one (Fig. 8). We fed the two reactors with

two identical syringes on the same syringe pump. For obtaining the same residence time in the two reactors, the length of the second microreactor was twice as the length of the first one (50 cm and 25 cm respectively). This set-up implies that the quantity of N435<sup>®</sup> beads in the second reactor is twice as the quantity in the first one, thus giving approximately the same rate of polymerization of VL as CL rate of polymerization is about twice as the rate for VL. So there is only small changes in conversion and molecular weight dispersity when going from  $[CL]/[I] = 40$ ,  $[VL]/[I] = 40$  (Table 4, entry 1) to  $[CL]/[I] = 40$ ,  $[VL]/[I] = 20$ , (Table 4, entry 2) and  $[CL]/[I] = 20$ ,  $[VL]/[I] = 40$  (Table 4, entry 3). In addition, experimental molecular weights were relatively close to the theoretical previewed ones proving the livingness of PCL produced and its effectiveness as a macro-initiator. In details, when using 40/40 equivalents respectively for CL and VL the conversion was 93% and the molecular weight dispersity  $\bar{D} = 1.26$  (entry 1). The much lower  $M_n$  value than theoretical value in entry 1 even though conversion reached 93% is explained by the presence of oligomers which are not recovered after precipitation. The monomer decrease measured by NMR combines monomer consumption by both polymer and oligomer formation. Thus, oligomers formation increases the determined conversion. When decreasing the VL equivalents to 40/20, the conversion increased to 97% (entry 2), whereas by decreasing the CL equivalents to 20/40, the conversion dropped to 88% (entry 3). This result show that living PCL is slightly less efficient as the PP initiator as the initiation of the CL and VL alone by PP in the same conditions led to the same conversion (Table S1, conv = 100% and S2, conv = 93% respectively).



**Fig. 8.** Immobilized enzymes in the integrated FEP tubing for enzymatic ring opening copolymerization.

**Table 4**

Enzymatic ring opening copolymerization of CL and VL in flow at 4 min of residence time for the two tubular reactors.<sup>a</sup>

Run	M (1)	M (2)	Eq.	$M_{n,theo} \times 10^{-3}$ (g/mol) <sup>a</sup>	$M_{n,NMR} \times 10^{-3}$ (g/mol) <sup>b</sup>	$\bar{D}^c$	Conv (%) <sup>a</sup>
1	CL	VL	40/40/1	8.1	5.8	1.26	93
2	CL	VL	40/20/1	6.4	5.7	1.32	97
3	CL	VL	20/40/1	5.8	6.1	1.24	88

<sup>a</sup> Calculated from  $(([M_1] + [M_2]/[I]) \times \text{Conv}) \cdot (M_w(\text{CL}) + M_w(\text{VL})) + M_w(\text{PP})$ .

<sup>b</sup> Determined by <sup>1</sup>H NMR.

<sup>c</sup> Determined by GPC.

\*M: Monomer \*Eq: molar equivalents

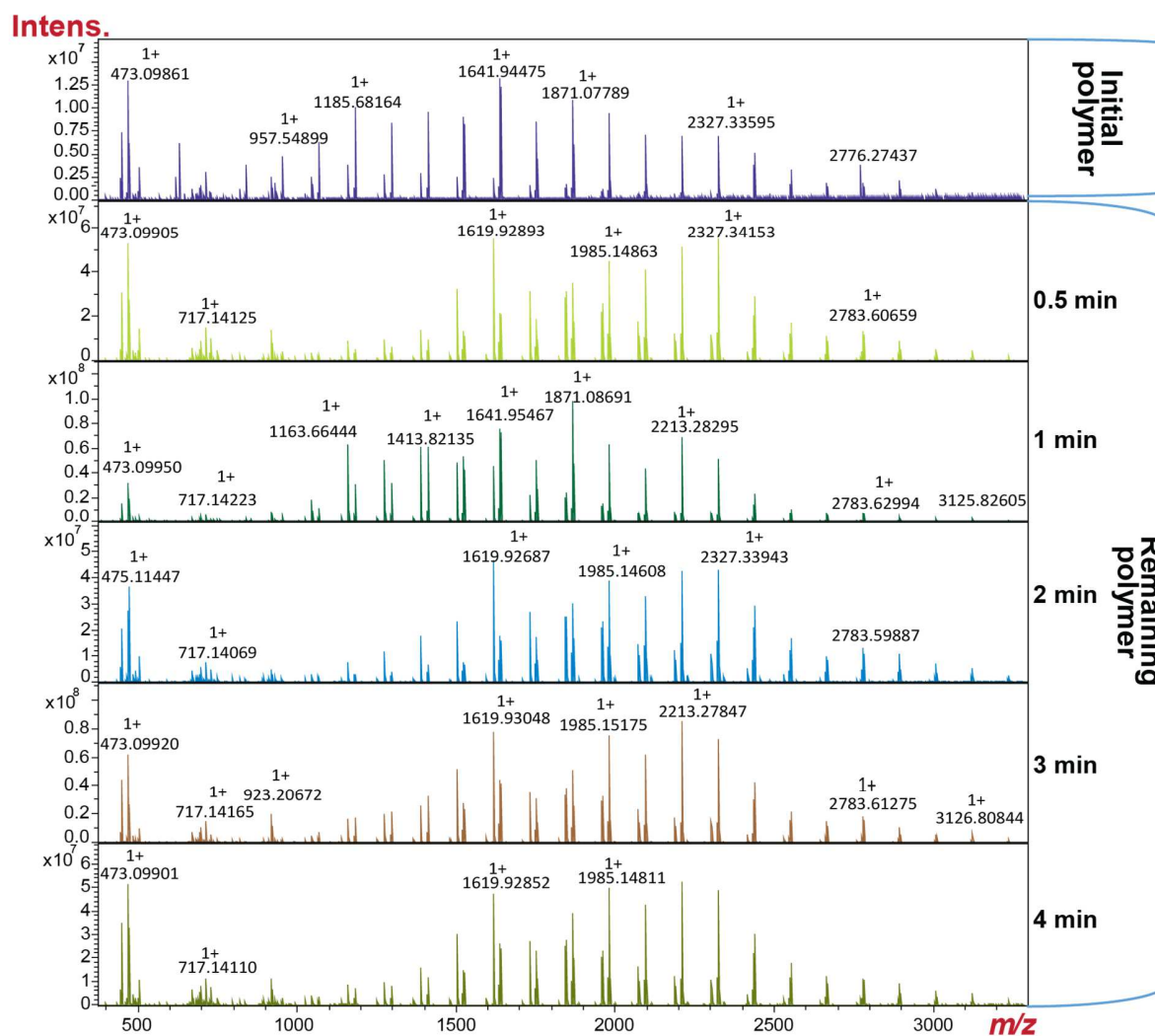
#### IV Degradation of polylactones by the action of N435® beads

We performed the degradation of PCL and PVL by the action of N435® beads (table S4). At 70 °C, 0.5 g of PCL were fed into the microreactor at flow rates affording

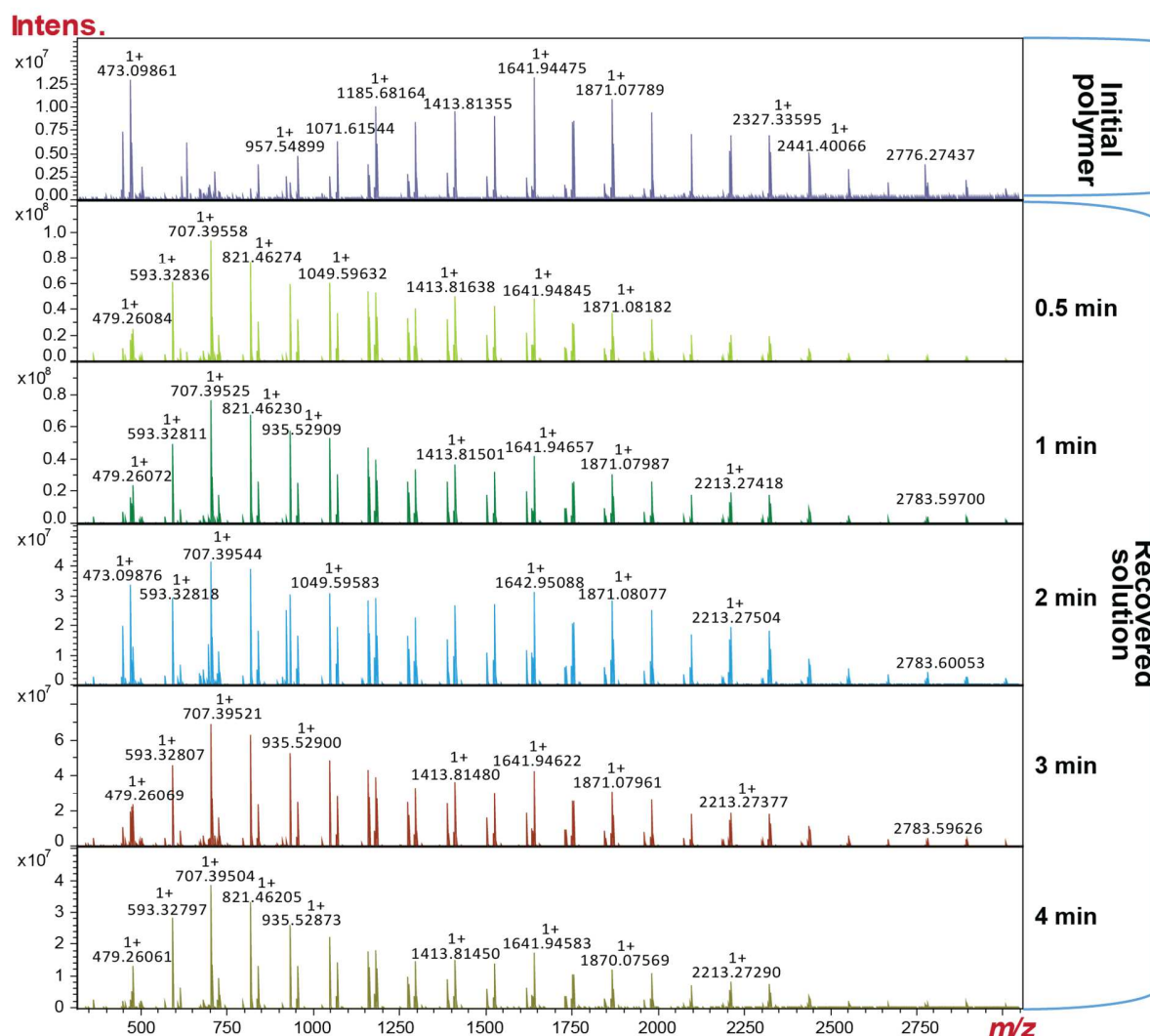
residence times from 0.5 to 4 min. The recovered solution was submitted to the same precipitation procedure in cold methanol as the starting PCL. We recovered for all residence times between 32% and 38% of the initial weight of the injected PCL (Table S4) which corresponds to 62 to 68% degradation. MALDI-FTICR spectra showed a high  $m/z$  distribution which was roughly similar to the intact polymer, but for the disappearance of the low  $m/z$  distribution (Fig. 9). Consequently, 62% to 68% of the PCL was degraded by the enzyme to oligomers that were not recovered by precipitation. MALDI-FTICR spectra presented in Fig. 10 showed that the highest peaks of the initial PCL (top spectrum, Fig. 10) is comprised between  $m/z$  1400 and 2400. After degradation and without precipitation of the polymer in the recovered solution (Fig. 10), the dominant peaks are shifted to smaller masses around  $m/z$  500 and 1000 which proves again the degradation of these polymers. After zooming (Figs. S18 and S19), we detected the presence of three different species, (1) polymer chains with initiator extremity, which are the dominant structure in the initial PCL, (2) macrocycles, the intensity of which increased after degradation and (3) chains with  $H_2O$  extremity, which are minority before and after degradation. We realized also the degradation of PVL at 60 °C, and it resulted in around 56%-70% of degradation (Table S4) in the same range as CL. The analyses MALDI-FTICR (Figs. S20, S21 and S22) supports also the same conclusions as for CL. In brief, the enzymes were able to degrade polylactones even with low residence time of 0.5 min.

The degradation of polylactones by lipase is already known, but in this study the degradation occurred in few minutes instead of several hours by other methods [46] even those including flow conditions [47]. This study shows the importance of choosing the optimum residence time to apply in order to prevent the degradation of the polymer formed along its synthesis.

*Poly lactones synthesis by enzymatic ring opening polymerization in flow*



**Fig. 9.** MALDI-FTICR MS of the initial PCLs prepared at 70 °C and those remained in their dried form after the degradation at 70 °C using different residence time in flow.



**Fig. 10.** MALDI-FTICR MS of the initial PCLs prepared at 70 °C and the recovered solution after the degradation at 70 °C using different residence time in flow.

## V Recycling of N435® beads

N435® beads immobilized into the tubular reactor were reused several times in the eROP of CL under identical conditions (residence time = 240 s and T = 70 °C). As shown in table S5, until reaction number 8, conversion was constantly higher than 96%, then decreased below 90% in reaction 18 and reached 77% in reaction 21. The molecular weights previewed by  $^1\text{H}$  NMR were in agreement with the experimental ones. This study proved the high efficiency of enzyme, which may be used about twenty

times in flow system without the need of any extra step or to recover the catalyst after each reaction. These results are in agreement with those obtained by Kundu et al [27] for flow lactone polymerization catalyzed by N435® beads. In their experiment the conversion dropped from 90% to 70% after twenty cycles of polymerization. In batch and under the same conditions, toluene at 70 °C, Poojari et al could recycle the N435® beads with maintaining constant the molecular weight of PCL for ten times which is the half of the cycles number that we reached in flow [48]. However, they found that in batch a disintegration of the resin particles occurred due to the stirring speed which was a limitation for the recovery and reuse of N435® beads. Furthermore, this mechanical disintegration induced CALB leaching. In our case, the reaction time was for 30 min (Eq. S2) for each cycle which is eight times less than the reaction time needed in batch (30 min in flow vs 4 h = 240 min in batch) which affected also the number of reuse cycles of N435® beads in batch.

## **VI Conclusion.**

In conclusion, we successfully produced PCL and PVL via e-ROP of CL and VL catalyzed by N435® beads in tubular reactor in flow. Under the best conditions at 70 °C and residence time of 240 s, the CL conversion was 100% with a molecular weight dispersity of  $\bar{D} = 1.3$ . In similar conditions (60°C, residence time = 214 s) VL afforded a conversion of 93% and a molecular weight dispersity of  $\bar{D} = 1.27$ . By increasing the residence time to 600 s both CL and VL were cleanly polymerized at room temperature, with a conversion of 94% and molecular weight dispersity of  $\bar{D} = 1.34$  for CL, and a conversion of 100%,  $\bar{D} = 1.28$  for VL. We verified that no conversion occurred without N435® beads neither at room temperature nor at 70 °C. As initiators, we found that unhindered primary alcohols are more efficient for the initiation than secondary alcohols. With hindered or bulky secondary alcohols or without added initiator, the initiations were done by residual water and the main products are macrocycles obtained with a large molecular weight dispersity [ $\bar{D} = 1.99$ -2.68] and molecular weight of [1600 – 2600 g/mol]. The only exception is cholesterol, a bulky secondary alcohol, which gave a sharper molecular weight dispersity [ $\bar{D} = 1.6$ ] presumably by interacting with CALB. Successful copolymerizations of CL and VL were done by sequential addition which



yielded copolymers of  $D_{\max} = 1.32$  and conversions up to 88%. We showed also the importance of choosing the optimum residence time in order to prevent the degradation of the formed polymers by the enzyme. Finally, under our conditions N435® beads kept a very good efficiency when the same beads packing in the microreactor more than twenty times without any regeneration. These results show that homemade tubular reactor readily assembled in the laboratory are efficient devices to produce polylactones by eROP. This setup paves the way to explore more stringent polymerization catalyzed by CALB such as the eROP of chiral lactones to obtain chiral polyesters [49]. This setup also opens the way to the study of enzymatic polycondensation in flow catalyzed by CALB, which is able to catalyze the transamidation of dimethyl 2,5-furandicarboxylate and heteroatom diamines [50]. The structure of the substrates, which modulates the activity of CALB to produce predominantly cyclic polyesters, deserves further studies.

#### **Declaration of competing interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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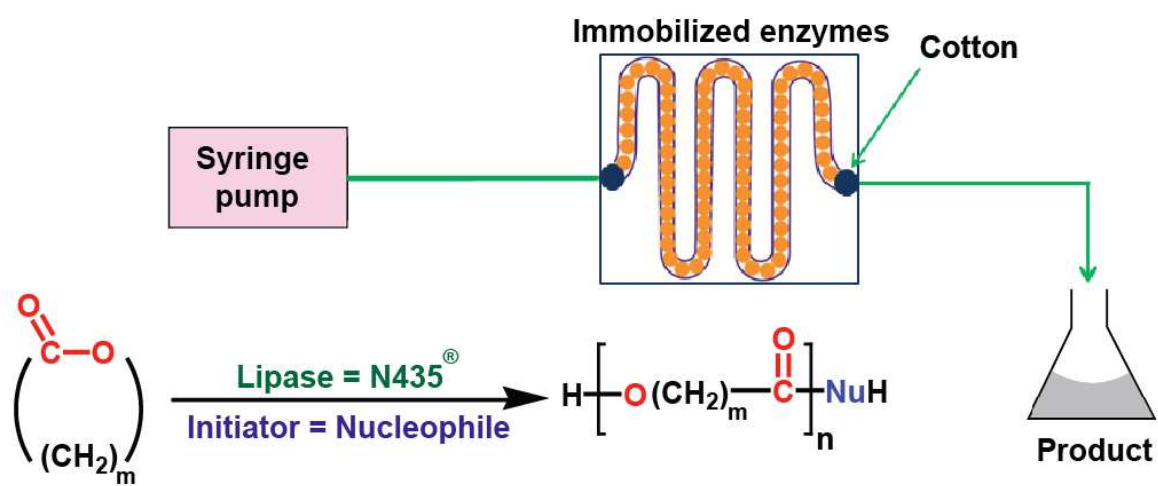
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Immobilized enzymes in FEP tubing for lactone e-ROP in flow.