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# 1 Polylactones synthesis by enzymatic ring opening 2 polymerization in flow

3

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11

12 **Abstract** Polycondensation in batch is the classical way to produce polyesters.

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

32

33 **Key Words**34 *Enzymatic Ring Opening Polymerization (e-ROP), Flow system, Polylactones, Novozym® 435*

35 **I Introduction**

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

49 Green chemistry has now become a very attractive field. It represents a new point of  
50 view in chemistry, promoting the use of green starting materials, solvents and process  
51 that respect the environment and afford products which are not damaging our  
52 environment [12]. In this paper, we focused on green synthesis of macromolecules.  
53 Enzymes; performing clean reactions with no side products and providing high  
54 selectivity with respect to enantio-, regio- and chemo-selectivities are the archetype of  
55 green catalysts. No conventional catalytic system affords such a degree of selectivity. In  
56 addition, enzymes are natural, renewable and nontoxic. Furthermore, enzymes are  
57 recyclable which allows their reuse multiple times [13-16]. The traceless green solvent,  
58 supercritical carbon dioxide ( $\text{scCO}_2$ ) can be used for promoting enzymatic  
59 polymerization [17]. Lipases are a class of enzymes which hydrolyse fatty acide esters  
60 and they are the most commonly used in enzymatic polymerization [15, 16]. Novozym  
61 435® from Novochem is a lipase B (CALB) immobilized on an acrylic resin which is  
62 commercially available [18]. Novozym 435® has been found the most effective enzyme  
63 to promote the ring opening polymerization of lactones [19].

64 Flow microreactors allow high performance organic syntheses. High surface to volume  
65 ratio of microreactors, short diffusion paths and more efficient heat and mass transfer  
66 compared with conventional platforms explain these improved performances [20-23].  
67 The reaction parameters are easily controlled. For example, the reaction time is  
68 adjusted by the simple variation of the flow rate or of the microchannel length. Polymer  
69 synthesis has also been developed in continuous flow microreactors [24, 25]. In a  
70 seminal work, Gross and Beers employed immobilized enzyme in continuous flow  
71 system to achieve  $\epsilon$ -caprolactone (CL) ring opening polymerization in toluene [26]. The  
72 polymerization took place in an Aluminium microchannel reactor coated with Kapton®  
73 film. In comparison with batch system, they obtained a higher number-average  
74 molecular weight ( $M_n$ ) of PCL in less reaction time proving the advantages of using  
75 N435® beads in a microreactor to catalyze the ROP of CL. Neither conversion nor  
76 chemoselectivity are affected when using toluene saturated with water [27]. These  
77 works have paved the way to several polylactones synthesis in micro-flow system [28-  
78 30]. In addition, Zhu et al. examined the chemoselectivity towards hydroxyl versus thiol  
79 functions via enzymatic ring opening polymerization of  $\delta$ -valerolactone (VL) in flow  
80 mode as well as its copolymerization where they obtained interesting results compared  
81 to the batch [31].  
82 In order to develop a green polyester synthesis, we combined the advantages of micro-  
83 flow system, ROP and green catalyst using a homemade setup easily built in any  
84 laboratory. CL and VL polylactones were synthesized as well as their copolymers via  
85 enzymatic ring opening. A comprehensive study of the parameters (initiators, flow rate,  
86 temperature and reaction time) of each reaction was performed. In this study, we were  
87 able to show the special behavior of cholesterol affording cyclic polyesters. The formed  
88 polymers were characterized by  $^1\text{H}$  nuclear magnetic resonance spectroscopy (NMR),  
89 mass spectrometry (MS) and gel permeation chromatography (GPC). Furthermore we  
90 studied the degradation of the formed polyesters to understand the kinetics and  
91 therefore to optimize the eROP. Our results show that the combination of enzymatic ring  
92 opening polymerization and micro-flow technology open the access to well-defined and  
93 cost effective polymer synthesis.

94 **II Methods and materials**

95 *II.1 Chemicals*

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

104

105 *II.2 Analytical methods*

106 Proton nuclear magnetic resonance spectra ( $^1\text{H}$  NMR) were recorded on an AVANCE  
107 300 spectrometer (300 MHz) (Bruker Biospin, Wissembourg, France) using  
108 tetramethylsilane (TMS) as the internal standard and using  $\text{CDCl}_3$  as solvent. The peak  
109 of non-deuterated chloroform resonates at 7.23 ppm. The experiments were carried out  
110 at 293 K.

111 Size exclusion chromatography (SEC) was performed on a Prominence chromatography  
112 chain (Shimadzu, Kyoto, Japon) fitted with a Refractive Index (RI) detector (RID-20A)  
113 and an UV detector (SPD-20A). The columns (KF-802 and KF-803L from Shodex  
114 (Tokyo, Japan)) were eluted with tetrahydrofuran (THF) at a flow rate of 1 mL/min at 30  
115 °C. The samples were previously prepared by dissolving 10 mg of sample in 1 mL THF.  
116 The solution was then filtered through a PTFE filter with a pore diameter of 0.45  $\mu\text{m}$ . A  
117 volume of 20  $\mu\text{L}$  was injected into the size exclusion chromatography to carry out the  
118 analysis. The SEC was calibrated with poly (styrene) standards. The number average  
119 molecular weight was determined from the UV detector absorbance.

120 Matrix assisted laser desorption ionization-time of flight mass spectra (MALDI-TOF MS)  
121 were recorded at 20 kV in positive ion mode on an ABISciex (Framingham, MA, USA)  
122 4800 plus mass spectrometer. Samples, NaI were dissolved in THF at a concentration  
123 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

124 volume ratio of 1:1:1. Then, 0.1  $\mu\text{L}$  of the final solution was spotted on the target plate.  
125 The mass range was set to  $m/z$  200–5000. The applied laser's power was 4500  
126 (arbitrary value) and 1000 laser shots were accumulated for each scan.

127 High resolution MALDI mass spectra were acquired on a SolariX XR FTICR instrument  
128 fitted with a 9.4 T actively shielded superconducting magnet and a dynamically  
129 harmonized cell (Bruker Daltonics, Bremen, Germany). This instrument was equipped  
130 with both laser desorption ionization source (Smartbeam® II, Nd:YAG harmonic 3 laser  
131 at 355 nm) and an electrospray source. Matrix-assisted laser desorption/ionization  
132 Fourier transform ion cyclotron resonance (MALDI-FT-ICR) mass spectra were acquired  
133 in positive ion mode. The mass range was set to  $m/z$  200–5000. The applied laser  
134 power was 20% and 500 laser shots were accumulated for each scan. Samples were  
135 deposited on the plate as previously described.

136

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

138 The enzyme immobilized tubular reactor was made from fluorinated ethylene propylene  
139 (FEP) tubing (i.d. 1.55 mm, length 25 or 50 cm, Cluzeau Info Labo (C.I.L.), Sainte-Foy-  
140 La-Grande, France) fitted with 8-mL stainless-steel syringe (thread 1/4–28 inch,  
141  $\varnothing = 1/16$  inch, PC5 702268, Harvard Apparatus) powered by a Harvard Apparatus  
142 (Holliston, MA, USA) PHD ULTRA XF syringe pump as shown in Fig. S1. The tube  
143 outlet was plugged by cotton to hold the enzymes beads after what the tube was filled  
144 with N435® beads. The enzyme bead load was 100 mg (10%w of CL) in the 25 cm long  
145 of FEP tube in which we performed the e-ROP of CL and 200 mg (20%w of VL) in the  
146 50 cm one for the VL e-ROP. The volume of the tubular reactor was estimated by  
147 flowing toluene through the reactor and estimated to be 0.28 and 0.55 mL, respectively  
148 for the 25 and 50 cm reactors. The stock solution lactone and initiator in dried toluene  
149 was prepared using Schlenk technique under nitrogen. All reactions were carried out  
150 following the same equivalents: 20/1/40 respectively for the monomer, the initiator and  
151 toluene (solvent). The mixture was transferred into the 8 mL stainless steel syringe  
152 under nitrogen atmosphere. The enzyme immobilized tubular reactor was flushed with  
153 dried toluene to remove the moisture and air. The stock solution was pumped into the  
154 tubular reactor at the desired flow rate, and the reactor was placed into a water bath at

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

162

163

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

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

178

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

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

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

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

191

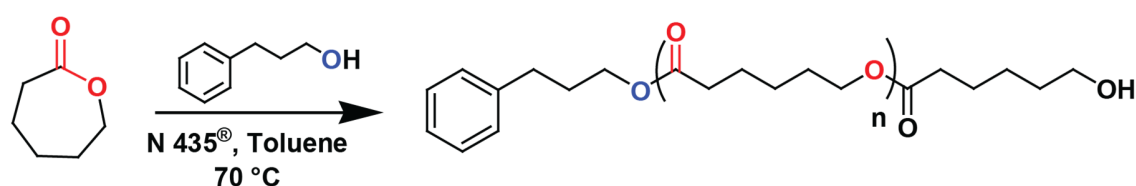
### 192 III Results and discussion

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

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

195 Following Gross et al.'s pioneering work [26, 27], we studied CL conversion versus  
196 residence time by e-ROP (Scheme 1) in flow in dry toluene using immobilized Novozym  
197 N435 (100 mg) and 3-phenyl-1-propanol as initiator with a monomer to initiator  
198 concentration ratio ( $[M]/[I]$ ) of 20 at a concentration of 4.5 M. The microreactor used in  
199 this study had approximately the same cross-section as the one used by Kundu et al.  
200 [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  
201 recent study Zhu et al [29] used a larger cross section of 11.34 mm<sup>2</sup> and a slightly  
202 longer length (300 mm).

203



204

205

Scheme 1. e-ROP of CL.

206

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



212 rate constant of  $0.004 \text{ s}^{-1}$  with a coefficient of correlation  $R^2$  of 0.98 (Fig. 2). This linear  
 213 correlation indicates a good control on the polymerization. During the propagation step  
 214 the concentration of active centers was not altered and no termination reactions  
 215 occurred [32]. In agreement with this kinetics, GPC showed a pretty narrow molecular  
 216 weight dispersity ( $\mathcal{D} = 1.35$ ). The MALDI-TOF spectrum (Fig. 3) presented a bell-shaped  
 217 peak distribution in agreement with GPC. A negligible presence of macrocycles was  
 218 also detected. We repeated the same study but at  $70 \text{ }^\circ\text{C}$ . As indicated in Table S1, the  
 219 conversion increased from 30% to 100% when the residence time was increased from  
 220 15 s to 240 s, and above 240 s the conversion remains at 100% (Fig. S4). So, at  $70 \text{ }^\circ\text{C}$   
 221 a lower residence time was enough to afford complete conversion. From the linear  
 222 kinetic plot presented in Fig. S5 a rate constant of  $0.011 \text{ s}^{-1}$  was obtained with a  
 223 coefficient of correlation  $R^2$  of 0.96. This first order kinetic rate constant is in agreement  
 224 with Kundu et al [26] who also found a rate constant of  $0.011 \text{ s}^{-1}$  for the same reaction.  
 225 GPC analysis showed that the molecular weight dispersity is slightly better at  $70 \text{ }^\circ\text{C}$  than  
 226 at  $20 \text{ }^\circ\text{C}$  (respectively  $\mathcal{D} = 1.30$  and  $\mathcal{D} = 1.35$ ). This molecular weight dispersity is better  
 227 than those Kundu et al [26] who showed the presence of oligomers and slightly higher  
 228 than the molecular-weight dispersity reported by Zhu et al [29] in approximately the  
 229 same conditions ( $\mathcal{D} = 1.12$  and  $1.17$  for  $([M]/[I])$  of 10 and 30). In all cases flow mode  
 230 provided lower molecular weight dispersity than batch since under the same conditions  
 231 Zhu et al [29] obtained PCL of  $\mathcal{D} = 1.48$  with a much higher reaction time of 14 h. As  
 232 shown below the molecular-weight dispersity is sensitive to the nature of the initiator  
 233 (see Table 3). The use of 6-mercapto-1-hexanol as initiator may explain their lower  
 234 molecular-weight dispersity. In the MALDI-TOF spectrum (Fig. S6) two other minor  
 235 species of PCLs were detected, chains initiated by  $\text{H}_2\text{O}$  and macrocycles which result  
 236 from their cyclisation, but the overwhelming majority remains PP end chains (Fig. 4).

237

238 **Table 1**

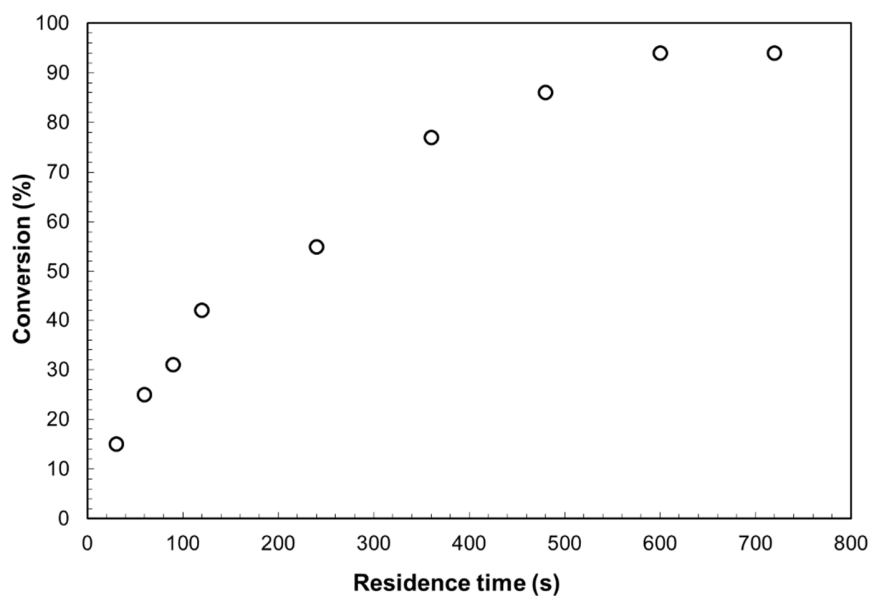
239 PCL produced by e-ROP in flow at room temperature.<sup>a</sup>

<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

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

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

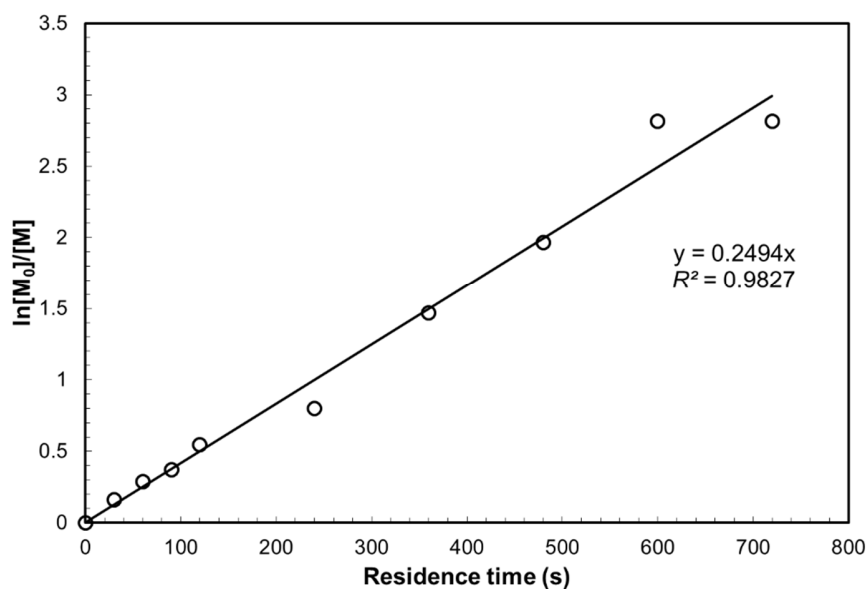
241 <sup>b</sup> Determined by <sup>1</sup>H NMR.



242

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

244

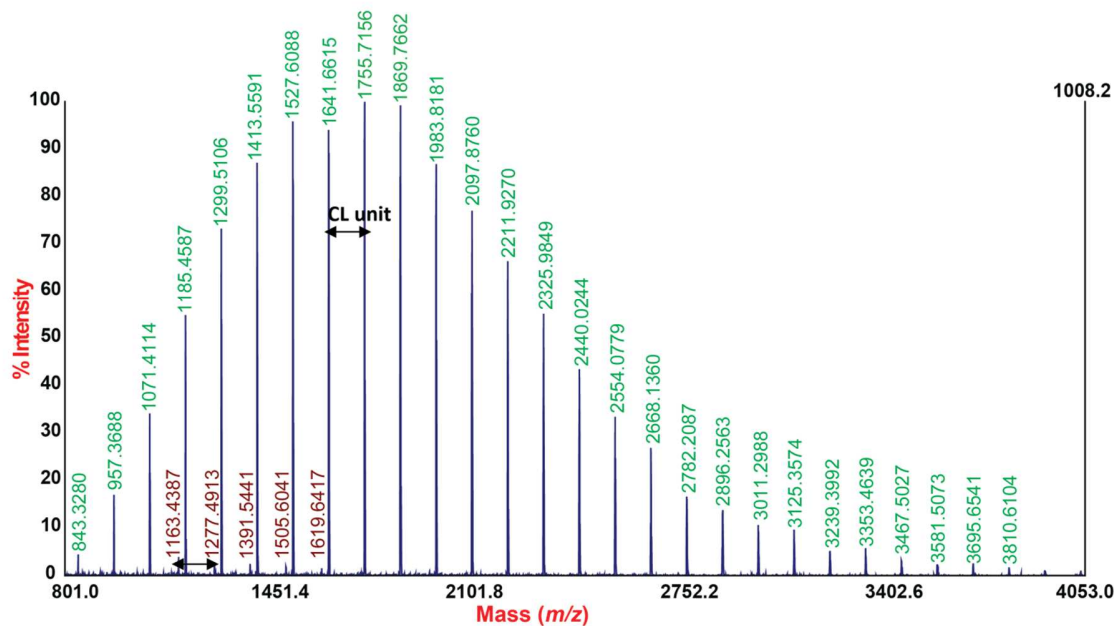


245

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

247

## Poly(lactones) synthesis by enzymatic ring opening polymerization in flow

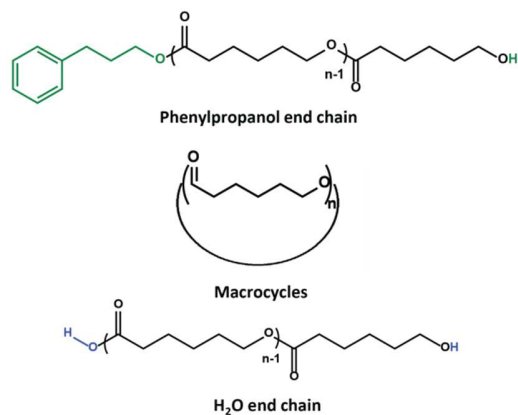


248

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

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

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



253

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

255

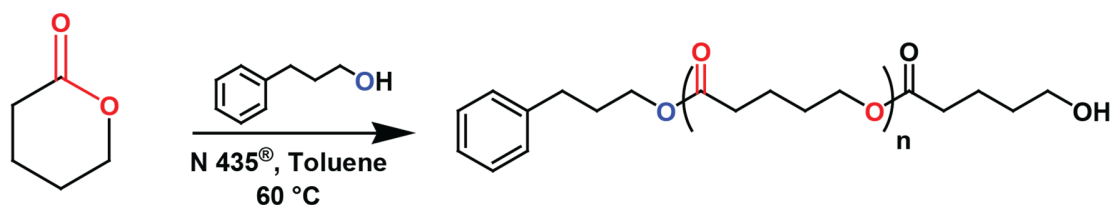
### 256 III.1.2 Poly( $\delta$ -valerolactone) synthesis.

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

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

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

261



262

263

**Scheme 2.** e-ROP of VL.

264

265 Table S2 shows that at 60 °C, the conversion of VL increased from 12% to 93% when  
266 the residence time was increased from 20 s to 214 s (Fig. S7). The linear relationship  
267 between the logarithm of the monomer concentration vs. residence time (s) indicates  
268 that the polymerization followed a first order kinetics. The rate constant and the  
269 coefficient of correlation  $R^2$  given by the slope are  $0.012 \text{ s}^{-1}$  and 0.96 respectively (Fig.  
270 S8). This rate constant, which is almost equivalent to the one for CL at 70 °C, was  
271 obtained by doubling the quantity of N435® beads. This result is in agreement with van  
272 der Mee et al. [34] who gave a Michaelis-Menten relationship in which the Michaelis  
273 constant  $K_M$  is almost the same for CL and VL but the catalytic rate constant  $k_{cat}$  is  
274 nearly the double for CL, leading to polymerization rates for CL that are twice as high.  
275 This linear relationship proved again the good control of the polymerization progress,  
276 also verified by GPC, which gave a good narrow molecular weight dispersity ( $\mathcal{D} = 1.27$ ).  
277 This value is slightly higher than those obtained by Zhu et al [31] in almost comparable  
278 reactions conditions ( $\mathcal{D} = 1.13$  and 1.16 for  $[M]/[I]$  of 10 and 30) using a larger cross  
279 section tube compared to us (11.34 mm<sup>2</sup> versus 1.9 mm<sup>2</sup> respectively) with a shorter  
280 length (300 mm versus 500 mm). However, a much higher residence time of 20 min  
281 was required to reach the highest conversions of 98%. In contrast, a residence time of  
282 214 s ( $\approx 3.5$  min) was enough in our case at  $[M]/[I] = 20$  to reach the highest conversion  
283 of 93%. The same reaction performed in batch system gave widely dispersed polymers  
284 ( $\mathcal{D} = 1.35$  for  $[M]/[I]$  of 30) for a higher reaction time of 600 min. The MALDI-TOF  
285 spectrum (Fig. S9) revealed the presence of two other families of species, chains  
286 initiated by H<sub>2</sub>O and macrocycles, although the dominant one still being the PP end

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

298

299 **Table 2**

300 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>	$\bar{D}$ <sup>d</sup>	Conv (%) <sup>c</sup>
1	480	1.9	2.5	-	97
2	600	2.3	2.0	1.28	100

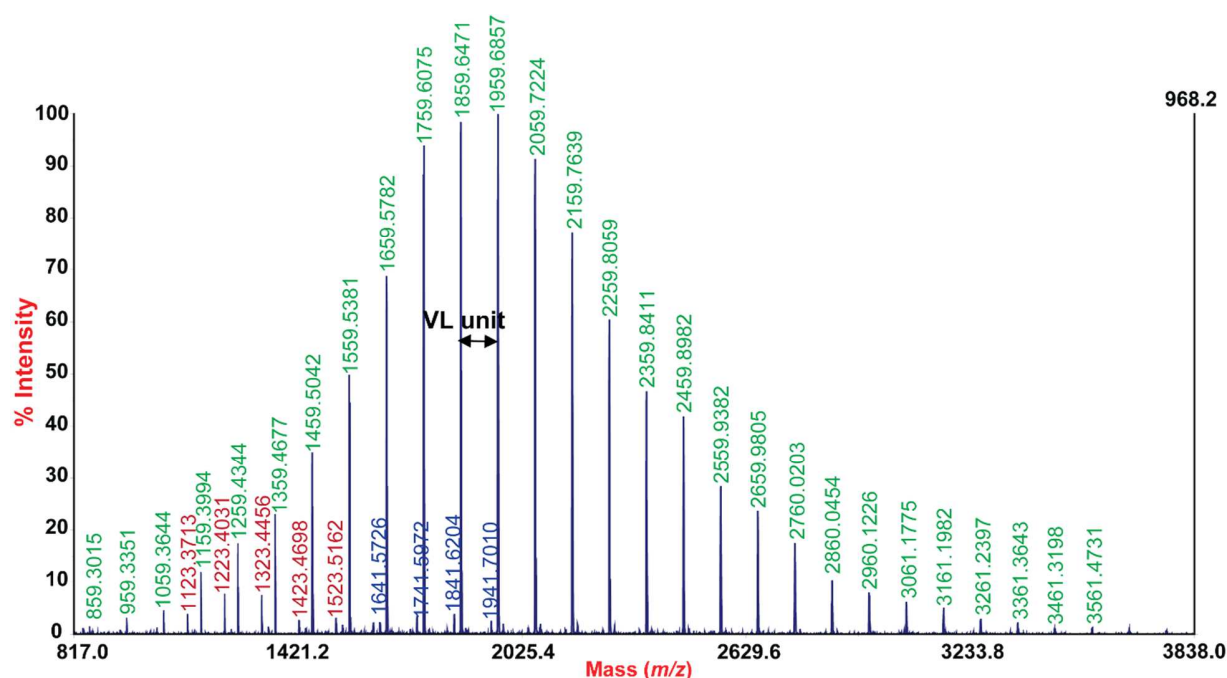
301 <sup>a</sup> [VL] = 4.5 M, [PP] = 0.225 M in toluene using 200 mg of N435® beads

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

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

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

305



306

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

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

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

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

312

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

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

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

352

353 **Table 3**

354 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>	$\bar{D}$ <sup>d</sup>	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

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

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

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

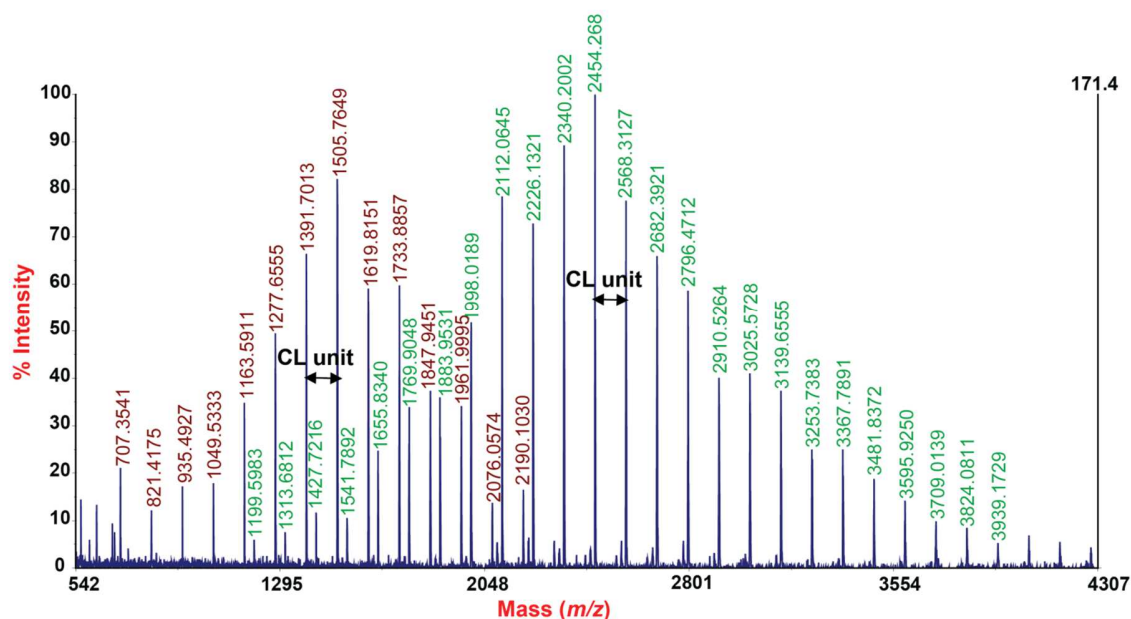
358 <sup>d</sup> Determined by GPC.

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

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



Polyactones synthesis by enzymatic ring opening polymerization in flow



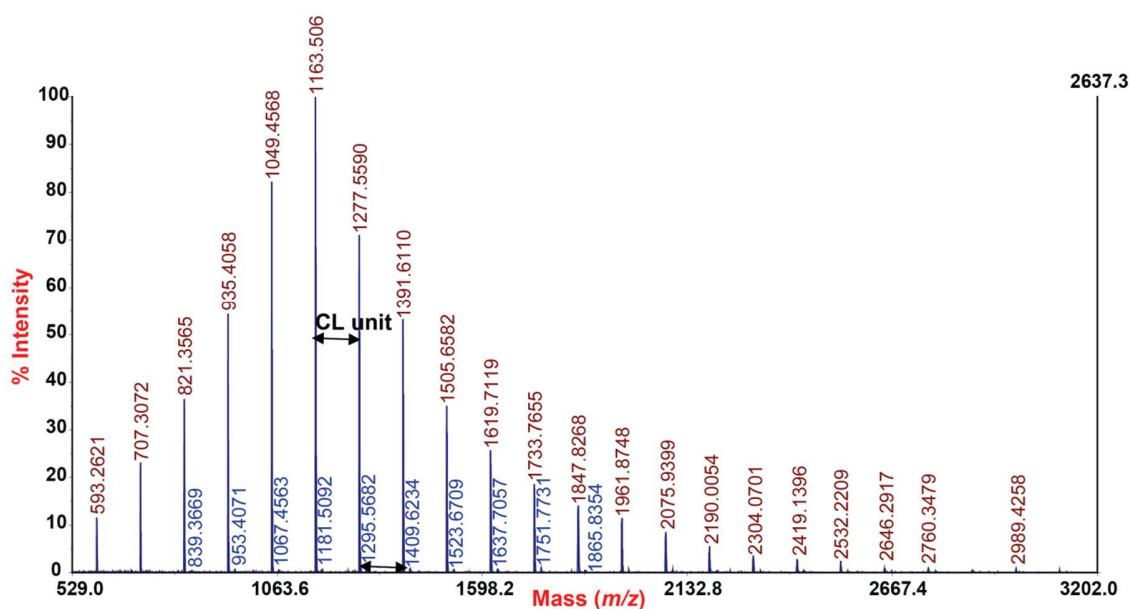
361

362 **Fig. 6.** MALDI-TOF MS of PCL initiated by 4-phenyl-2-butanol in flow.

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

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

365



366

367 **Fig. 7.** MALDI-TOF MS of PCL initiated by cholesterol in flow.

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

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

370 In view of the results obtained with cholesterol, we carried out the e-ROP of CL at 70°C  
371 in the absence of added initiator. As shown in Table S3, we obtained 100% of  
372 conversion using 100 mg of N435® beads at a residence time of 120 s or 240 s (S3,  
373 entries 1 and 2). In addition, 44% of conversion was obtained at room temperature in  
374 240 s of residence time (Run 3). Increasing the quantity of N435® beads to 200 mg at a  
375 residence time of 240 s gave the same results showing that the obtain polymer is  
376 resistant to depolymerization (Run 4). Unsurprisingly the polymers had the appearance  
377 of cotton exactly like those obtained when using cholesterol as an initiator. MALDI-TOF  
378 spectra also indicate the presence of macrocycles only (for an example of MALDI-TOF  
379 spectrum see Fig. S17). Nevertheless, the GPC of these polymers showed a much  
380 wider molecular weight dispersity [ $D = 1.99-2.68$ ]. Epoxidized cholesterol is specifically  
381 acetylated in presence of vinylacetate by N435® beads, showing that cholesterol is a  
382 substrate for immobilized CALB [42]. In other words, even if it not directly implied in  
383 lactone polymerization, cholesterol modulates the CALB activity. The maximum CALB  
384 which can be immobilized on Lewatit VP OC 1600 support is 30 mg per gram [43]. As  
385 the molecular weight of CALB is around 33 kDa the quantity of CALB enzyme in the  
386 reactor which contains 100 mg or 200 mg of N435® beads was around 100 pmol for CL  
387 polymerization and 200 fmol for VL polymerization. The quantity of cholesterol in the  
388 reactor was 63  $\mu\text{M}$  and 126  $\mu\text{M}$  for CL and VL respectively. Hence, cholesterol is  
389 present in a more than 100-fold excess compared to CALB. CALB has a stereospecific  
390 pocket for secondary alcohols [44] and the non-reacting enantiomer during kinetic  
391 resolution modulates its reactivity [45], which may explain the effect of a large excess of  
392 cholesterol. Another explanation is the complexation of cholesterol to the surface of the  
393 Lewatit VP OC 1600 support and the modification of the hydrophobicity of the enzyme  
394 environment, which also modulates its reactivity [43].

395

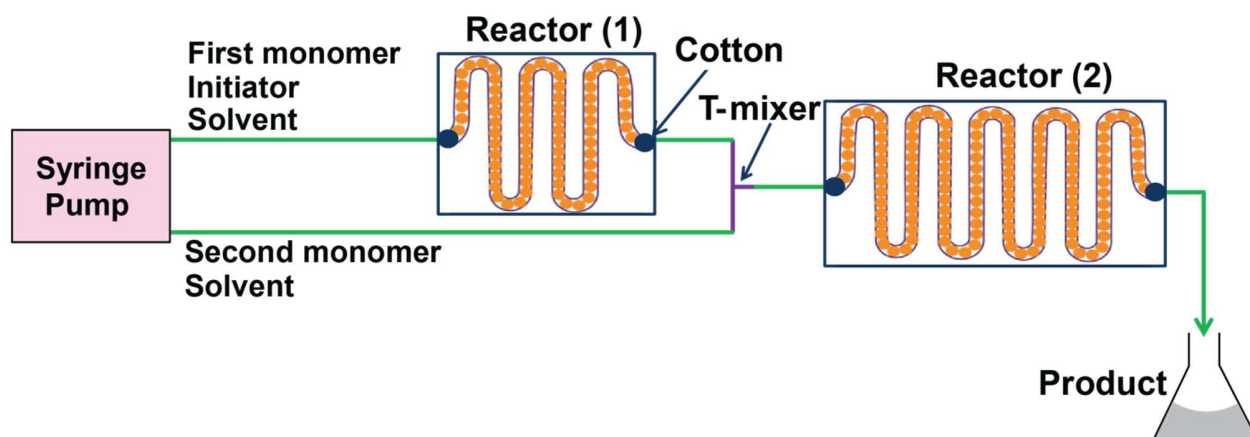
### 396 *III.3 Enzymatic ring opening copolymerization (e-ROCP) of lactones to produce di-block* 397 *copolymer in the integrated microreactor system by sequential addition*

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

401 two identical syringes on the same syringe pump. For obtaining the same residence  
402 time in the two reactors, the length of the second microreactor was twice as the length  
403 of the first one (50 cm and 25 cm respectively). This set-up implies that the quantity of  
404 N435<sup>®</sup> beads in the second reactor is twice as the quantity in the first one, thus giving  
405 approximately the same rate of polymerization of VL as CL rate of polymerization is  
406 about twice as the rate for VL. So there is only small changes in conversion and  
407 molecular weight dispersity when going from [CL]/[I] = 40, [VL]/[I] = 40 (Table 4, entry 1)  
408 to [CL]/[I] = 40, [VL]/[I] = 20, (Table 4, entry 2) and [CL]/[I] = 20, [VL]/[I] = 40 (Table 4,  
409 entry 3). In addition, experimental molecular weights were relatively close to the  
410 theoretical previewed ones proving the livingness of PCL produced and its effectiveness  
411 as a macro-initiator. In details, when using 40/40 equivalents respectively for CL and VL  
412 the conversion was 93% and the molecular weight dispersity  $\bar{D} = 1.26$  (entry 1). The  
413 much lower  $M_n$  value than theoretical value in entry 1 even though conversion reached  
414 93% is explained by the presence of oligomers which are not recovered after  
415 precipitation. The monomer decrease measured by NMR combines monomer  
416 consumption by both polymer and oligomer formation. Thus, oligomers formation  
417 increases the determined conversion. When decreasing the VL equivalents to 40/20,  
418 the conversion increased to 97% (entry 2), whereas by decreasing the CL equivalents  
419 to 20/40, the conversion dropped to 88% (entry 3). This result show that living PCL is  
420 slightly less efficient as the PP initiator as the initiation of the CL and VL alone by PP in  
421 the same conditions led to the same conversion (Table S1, conv = 100% and S2, conv  
422 = 93% respectively).

423

Poly lactones synthesis by enzymatic ring opening polymerization in flow



424  
425 **Fig. 8.** Immobilized enzymes in the integrated FEP tubing for enzymatic ring opening copolymerization.  
426

427 **Table 4**

428 Enzymatic ring opening copolymerization of CL and VL in flow at 4 min  
429 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

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

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

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

433 \*M: Monomer \*Eq: molar equivalents

434

435 **IV Degradation of polylactones by the action of N435® beads**

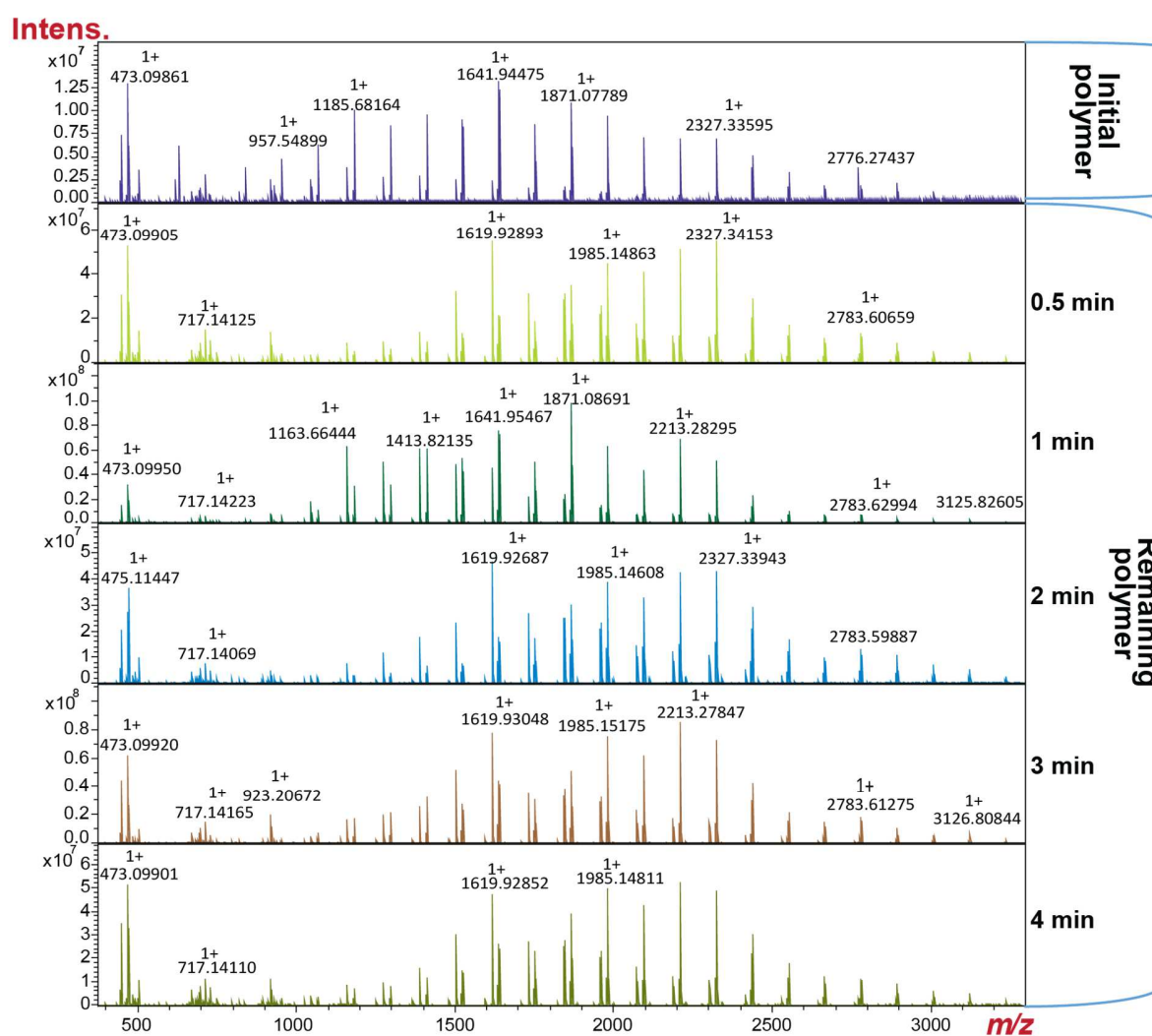
436 We performed the degradation of PCL and PVL by the action of N435® beads (table  
437 S4). At 70 °C, 0.5 g of PCL were fed into the microreactor at flow rates affording

438 residence times from 0.5 to 4 min. The recovered solution was submitted to the same  
439 precipitation procedure in cold methanol as the starting PCL. We recovered for all  
440 residence times between 32% and 38% of the initial weight of the injected PCL (Table  
441 S4) which corresponds to 62 to 68% degradation. MALDI-FTICR spectra showed a high  
442  $m/z$  distribution which was roughly similar to the intact polymer, but for the  
443 disappearance of the low  $m/z$  distribution (Fig. 9). Consequently, 62% to 68% of the  
444 PCL was degraded by the enzyme to oligomers that were not recovered by  
445 precipitation. MALDI-FTICR spectra presented in Fig. 10 showed that the highest peaks  
446 of the initial PCL (top spectrum, Fig. 10) is comprised between  $m/z$  1400 and 2400.  
447 After degradation and without precipitation of the polymer in the recovered solution (Fig.  
448 10), the dominant peaks are shifted to smaller masses around  $m/z$  500 and 1000 which  
449 proves again the degradation of these polymers. After zooming (Figs. S18 and S19), we  
450 detected the presence of three different species, (1) polymer chains with initiator  
451 extremity, which are the dominant structure in the initial PCL, (2) macrocycles, the  
452 intensity of which increased after degradation and (3) chains with H<sub>2</sub>O extremity, which  
453 are minority before and after degradation. We realized also the degradation of PVL at  
454 60 °C, and it resulted in around 56%-70% of degradation (Table S4) in the same range  
455 as CL. The analyses MALDI-FTICR (Figs. S20, S21 and S22) supports also the same  
456 conclusions as for CL. In brief, the enzymes were able to degrade polylactones even  
457 with low residence time of 0.5 min.

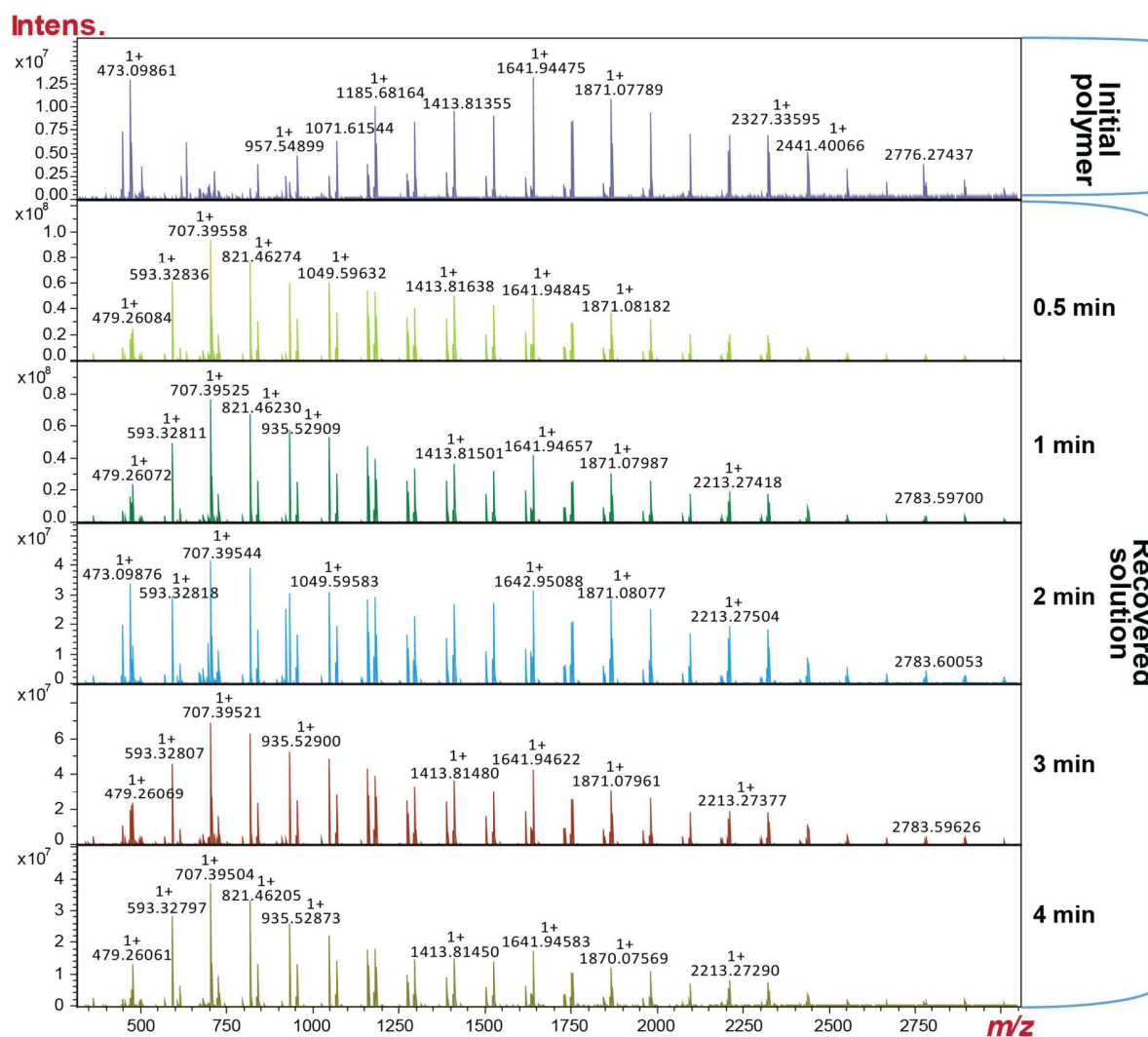
458 The degradation of polylactones by lipase is already known, but in this study the  
459 degradation occurred in few minutes instead of several hours by other methods [46]  
460 even those including flow conditions [47]. This study shows the importance of choosing  
461 the optimum residence time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

463

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



464  
 465 **Fig. 9.** MALDI-FTICR MS of the initial PCLs prepared at 70 °C and those remained in their dried  
 466 form after the degradation at 70 °C using different residence time in flow.



467  
 468 **Fig. 10.** MALDI-FTICR MS of the initial PCLs prepared at 70 °C and the recovered solution after  
 469 the degradation at 70 °C using different residence time in flow.  
 470

## 471 **V Recycling of N435® beads**

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

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

491

## 492 **VI Conclusion.**

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



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

521

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

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

525

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536

537

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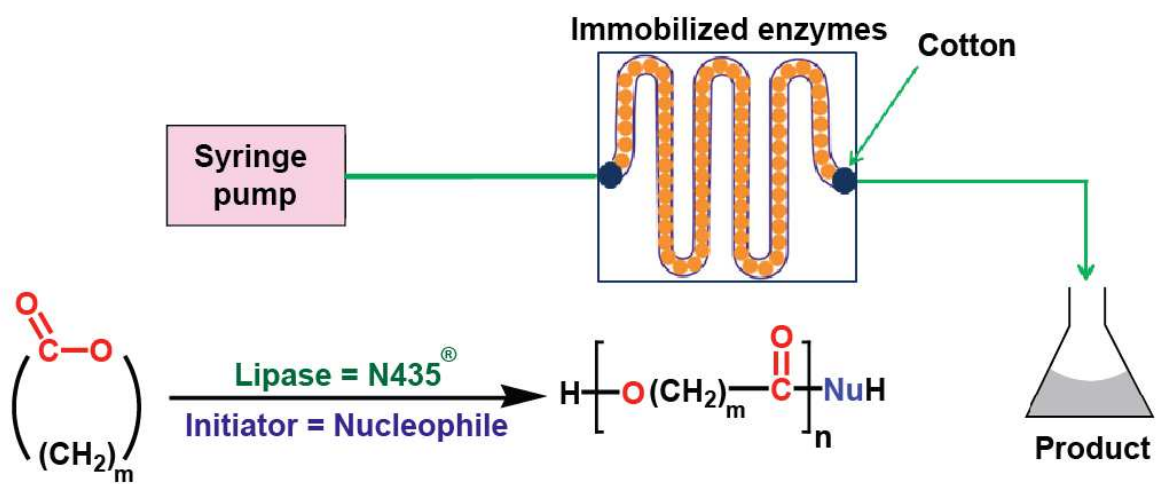
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Immobilized enzymes in FEP tubing for lactone e-ROP in flow.