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- Stéphane W. Duchiron, Éric Pollet, Sébastien Givry, Luc Avérous, "Mixed systems to assist enzymatic ring opening polymerization of lactide stereoisomers", *IVth Sino-French Symposium on Polymers and Soft Matter*, **2015**/10/12-14, Strasbourg, France
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Liste des abréviations

PLA : acide polylactique
PBS : poly(butylène succinate)
PHA : polyhydroxyalkanoate
CL : *ε-caprolactone*
PCL : poly(*ε-caprolactone)*
TCL : *ε-thiocaprolactone*
PTCL : poly(*ε-thiocaprolactone)*

TEA : triéthylamine
DMAP : 4-dimethylaminopyridine

Ser : Serine
His : Histidine
Glu : Glutamate
Asp : Aspartate
Met : Methionine
Cys : Cysteine
Boc : *tert*-butoxycarbonyle
N-Boc Cys HE : N-Boc -cysteine hexyl ester

ROP : polymérisation par ouverture de cycle
eROP : polymérisation enzymatique par ouverture de cycle

LBC : Lipase de *Burkholderia cepacia*
LCR : Lipase de *Candida rugosa*
CALB : Lipase B de *Candida antarctica*
CALBim : Lipase B de *Candida antarctica* immobilisée
N435 : Novozyme 435 (Lipase B de *Candida antarctica* immobilisée sur résine acrylique)
PPL : Lipase pancreatic porcine

SEC : chromatographie d'exclusion stérique
NMR : résonance magnétique nucléaire
TGA : Analyse thermogravimétrique
MALDI : source d'ionisation laser assistée par une matrice
ToF : analyseur de temps de vol
MS : spectroscopie de masse
FTIR : spectroscopie infrarouge a transformé de Fourrier

I. Introduction

Ces dernières années, une large réflexion sur l'impact environnemental de nos modes de vie actuels s'est engagée. Cette réflexion se traduit par un intérêt et une volonté accrue de « détoxifier » nos écosystèmes et d'assainir notre société, aussi bien d'un point de vue individuel que sociétal.

Afin de limiter la quantité de produits potentiellement toxiques dans notre environnement les pratiques actuelles dans le secteur des matériaux et de chimie sont en train d'évoluer très rapidement, une chimie dites « verte » est en plein développement catalysée par une prise de conscience sociétale. La chimie verte repose sur 12 principes fondateurs[1], qui sont donné en Annexe 1, parmi lesquels on trouve notamment la recherche de procédés et de produits pas ou peu toxiques pour l'homme et son environnement (troisième et quatrième principe), l'utilisation de ressources renouvelables (septième principe) ou encore l'utilisation préférentielle de procédés catalytiques (neuvième principe). Cette philosophie s'applique aussi bien à la chimie dite « traditionnelle » qu'a la biochimie ou à la chimie des polymères et matériaux.

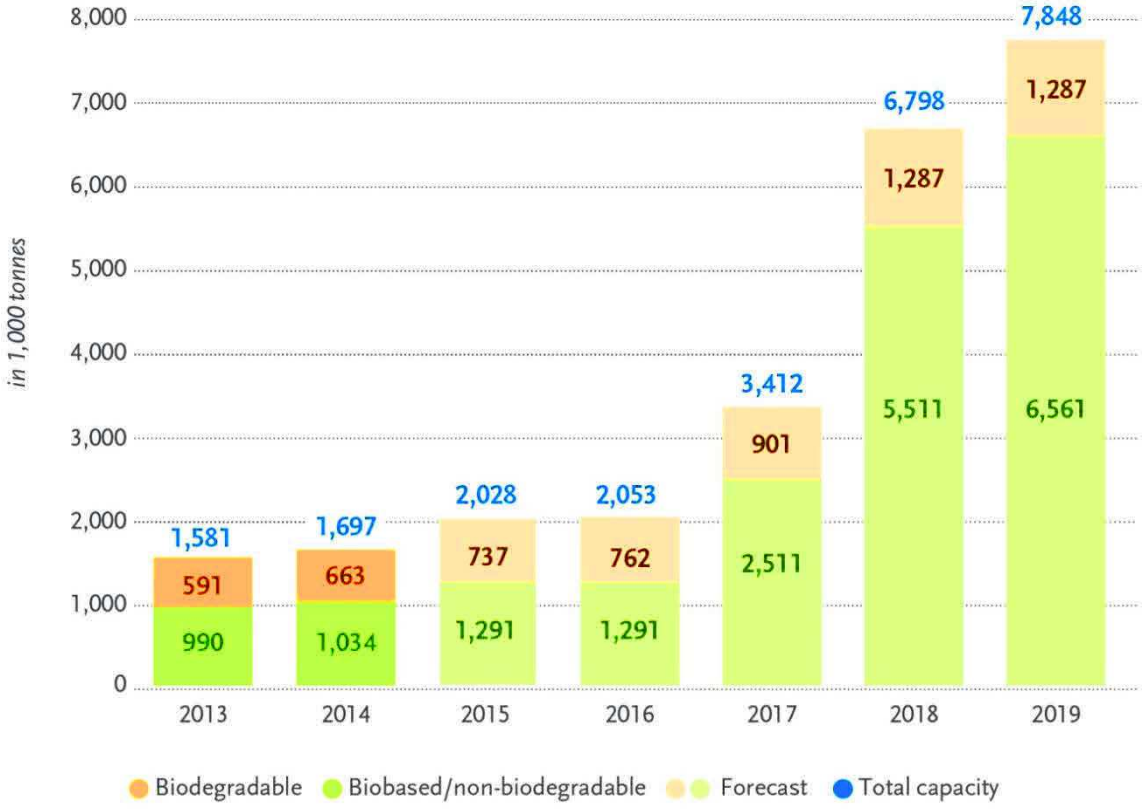


***Figure I-1**. Projection de l'évolution de la capacité de production mondiale en bioplastiques.*

Source : European Bioplastics, Institute for Bioplastics and Biocomposites, Nova-institute (2015)[2]

L'application du septième principe favorise le développement des polymères biosourcés, qui connaissent, depuis quelques années, un développement rapide, avec une capacité de production mondiale qui est passée de 180 milliers de tonnes en 2008 à plus de 2 millions de tonnes en 2015 (Figure 1), soit une capacité de production multipliée par plus de 11 en 7 ans pour un marché global qui s'exprime actuellement en milliards de d'euros. La demande mondiale croissante en bioplastiques a permis le développement d'un certain nombre de matériaux, comme l'acide polylactique (PLA), l'amidon thermoplastique ou encore le poly(butylène succinate) (PBS). Cependant aujourd'hui, les polymères biosourcés sont loin de pouvoir se substituer largement à leurs équivalents pétrosourcés soit pour des raisons de coût soit parce qu'ils n'atteignent pas les caractéristiques requises pour être en adéquation avec des applications où la performance est recherchée. Malgré cela, pour répondre à l'explosion à venir de la demande, qui d'après les données du Nova Institute (figure 1) pourrait être multipliée par quatre d'ici 2019, la recherche dans ce domaine se développe

rapidement, permettant d'envisager de nouvelles solutions de remplacement pour de nombreux matériaux pétrosourcés à court ou à moyen terme.

Il est à noter que la production actuelle des polymères biosourcés au niveau industriel est loin de suivre tous les principes de la chimie verte. En effet par exemple, de nombreux catalyseurs organométalliques sont actuellement utilisés comme par exemple l'octoate d'étain, qui est principal catalyseur pour la synthèse de l'acide polylactique (PLA). Ce catalyseur est allergène, nocif pour l'environnement aquatique et est également suspecté d'être reprotoxique. C'est pourquoi la synthèse enzymatique de polymères biosourcés retient une attention particulière au niveau académique et industriel, ces dernières années.

Les enzymes sont les catalyseurs du vivant. En effet, la plupart des réactions chimiques au sein des êtres vivants sont catalysées par une enzyme ou une combinaison d'enzymes. Ainsi, on trouve des enzymes capables de catalyser la plupart des réactions chimiques.

Les enzymes de par leurs actions constituent donc une alternative potentielle à de nombreux catalyseurs conventionnels issus d'une chimie traditionnelle. Ainsi, l'application des troisième, sixième et neuvième principes de la chimie verte a conduit à de nombreux travaux visant à substituer certains catalyseurs organiques et organométalliques, souvent toxiques, par des enzymes[3] qui sont potentiellement très sélectives, pour la plupart non-toxiques et qui sont efficaces dans des condition très douces contrairement à de nombreux catalyseurs chimiques. C'est la raison pour laquelle la catalyse enzymatique est un thème scientifique actuellement en plein développement dans de nombreux laboratoires dans le monde, notamment dans le domaine de la synthèse de polymères, avec une production croissante d'articles qui atteint environ 300 pour 2015

Dans cette étude, effectuée en partenariat avec le groupe français Soufflet, nous étudierons la catalyse enzymatique appliquée à la polymérisation par ouverture de cycle (enzymatic Ring Opening Polymerization, eROP). L'intérêt de ce type de synthèse est qu'elle est très peu toxique pour l'homme et pour son environnement, mais aussi elle moins énergivore puisque les réactions enzymatique se font naturellement à basse température par rapport aux systèmes catalytiques traditionnels.

Contexte industriel du projet

Ce projet de recherche s'inscrit aussi dans les programmes de recherche et de développement du groupe Soufflet. Il s'agit d'une entreprise agro-industrielle de dimension internationale comptant environ 7500 employés répartis dans 18 pays. Les cœurs de métier du groupe sont la collecte et la première transformation des céréales. Toutefois, ces dernières années le groupe a largement diversifié ses activités, vers l'agroalimentaire mais également vers les services aux agriculteurs et horticulteurs. C'est dans ce contexte de développement important que le groupe a mis en place une vraie politique de valorisation de ses coproduits, d'une part avec des projets d'économie d'énergie via des chaudières à biomasse et d'autre part avec une politique de Recherche & Développement ambitieuse centrée sur un ensemble de technologies de fermentation en milieu solide comme en milieu liquide permettant la production d'enzymes et celle de synthons d'intérêt.

Ainsi, le groupe Soufflet a entamé différents programme de recherches à destination des filières amont (avec des programmes de bioprotection des plantes, ou d'optimisation des sols) ou des filières aval (ingrédient pour l'industrie agroalimentaire, alimentation animal) à ses cœurs de métier.

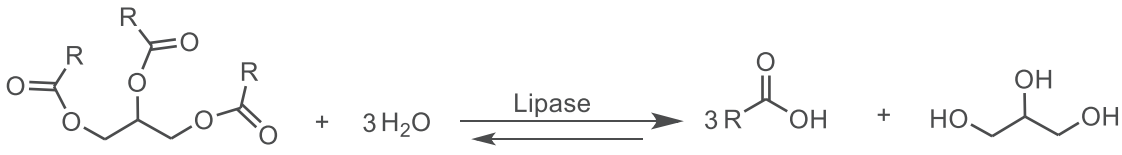
Ce projet de recherche s'insère donc à la fois dans la recherche de nouvelles voies de valorisation des enzymes produites par le groupe, mais également dans une optique de valorisation de synthons pouvant être produit par fermentation des coproduits du groupe Soufflet tel que l'acide lactique ou certain acides aminés.

Toutefois, les enzymes disponibles ainsi que les synthons déjà utilisés par le groupe ont défini le cadre initial de l'étude. En effet, Soufflet, par l'intermédiaire de sa filiale Lyven, produit déjà des protéases et a mis en place un programme de recherche sur les lipases (à destination de l'industrie boulangère).

Les familles de monomères susceptibles d'être polymérisés par les enzymes produites par le groupe, à savoir la polymérisation de lactones et dilactones comme le lactide ou le glycolide par des lipases ou protéases, ainsi que les programmes de recherche visant à produire des synthons ou molécules « plateforme », tel que l'acide lactique, ont donc naturellement orienter notre étude vers la synthèse enzymatique de PLA.

Contexte scientifique

Les lipases font partie des triacylglycérol hydrolases (E.C. 3.1.1.3). Ce sont des hydrolases à serine. En raison de leur rôle biologique naturel, qui est l'hydrolyse des triglycerides (Figure 2), les lipases, dans leurs conditions biologiques, fonctionnent généralement aux interfaces lipide-aqueuse. L'enzyme étant généralement hydrosoluble elle doit comporter de larges zones lipophiles afin de pouvoir accueillir son substrat. Pour les mêmes raisons, afin que le substrat soit stable dans le site actif, ce dernier doit également présenter de larges zones apolaires permettant de stabiliser le triglycéride via des interactions lipidiques.



R = chaine grasse

Figure I-2. *fonction naturelle des lipases : hydrolyse des triglycérides*

Les propriétés de lipophilie et d'activité interfaciale des lipases en font des enzymes de choix pour la chimie organique. En effet, ces enzymes sont relativement stables dans des solvants apolaires tels que ceux classiquement utilisés dans les réactions chimiques.

La réaction d'hydrolyse des fonctions esters est une réaction réversible. Les lipases sont à la fois capable de rompre des liaisons esters ou d'en former, en fonction de la quantité d'eau présente dans le milieu réactionnel. De ce fait, les lipases sont depuis longtemps utilisées en chimie comme catalyseurs d'estérification et de transestérification. Plus récemment ces enzymes ont fait l'objet d'une attention particulière comme alternative aux catalyseurs chimiques de synthèse des polyesters.

D'un point de vue chimique, la polymérisation par ouverture de cycles (ROP) des lactones est une forme particulière de transestérification. Les lipases sont utilisées pour synthétiser des polymères depuis le milieu des années 80[4,5] et le cas particulier de la ROP est étudié depuis le début des années 90[6].

La polymérisation enzymatique par ouverture de cycle des lactones a été beaucoup étudiée ces dernières années, particulièrement dans le cas de la ε-caprolactone[7,8]. Les nombreuses études de cette réaction ont permis d'établir un mécanisme probable (Figure 3) et de comprendre certains des paramètres clef de cette réaction, comme par exemple la quantité d'eau. Celle-ci sera un paramètre important à considérer dans le cas de la synthèse d'un polyester puisque les lois sur les équilibres chimique nous disent que sa minimisation est indispensable au déplacement de l'équilibre vers la formation du polyester, mais le catalyseur enzymatique nécessite une certaine quantité d'eau pour ne pas être dénaturé et donc inefficace. Ainsi, la recherche de la quantité d'eau optimale doit être considérée très attentivement[9,10].

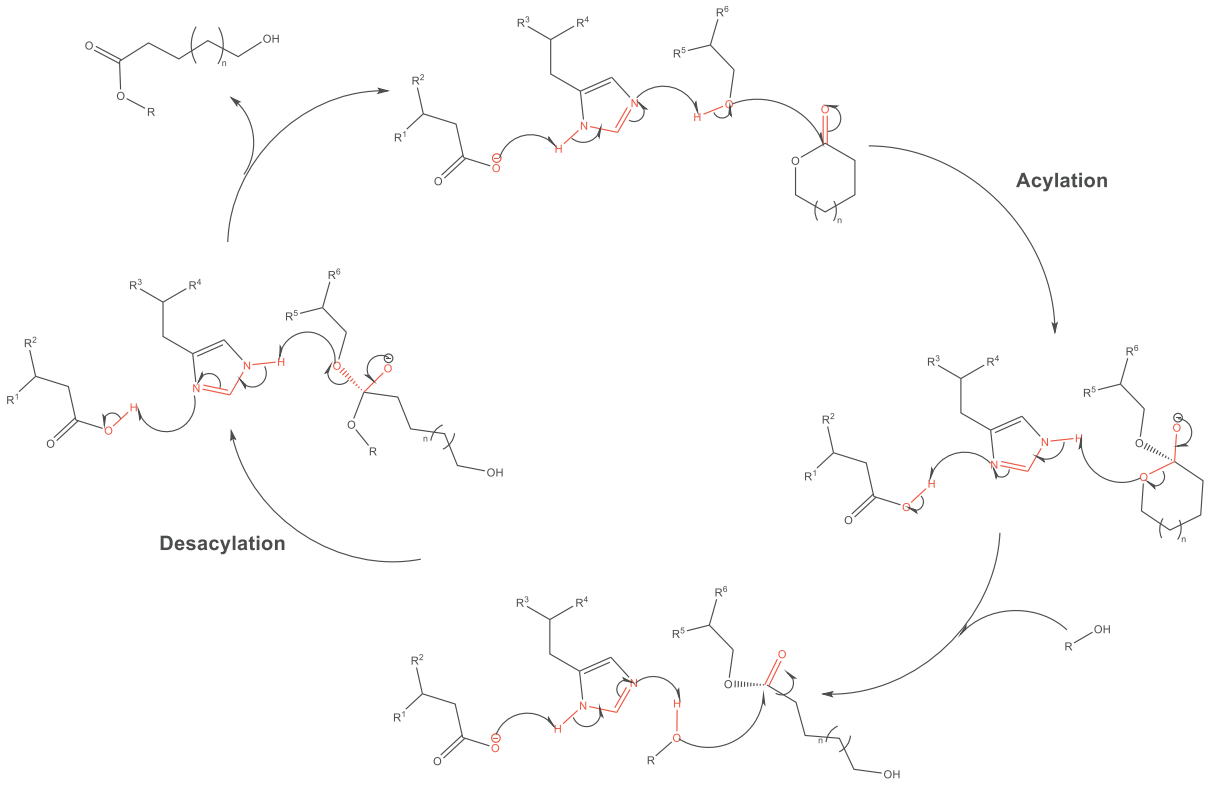


***Figure I-3**. mécanisme de polymérisation par ouverture de cycle de lactones catalysé par une lipase, adapté du mécanisme proposé par S. Kobayashi[11]*

D'autres paramètres ont été étudiés aussi bien au niveau de l'enzyme (nature de l'enzyme, structure de celle-ci[12], influence de l'immobilisation et du support d'immobilisation), qu'au niveau de la réaction elle-même (solvant, température, hydratation du milieu[9,10],

concentration des réactifs et du catalyseur) ou encore au niveau de la structure du monomère (taille de cycle, polarité, substituant, stéréochimie, hétéroatome intra-cyclique).

Par exemple, certains auteurs ont étudié l'influence du type de solvants, notamment en fonction de leur valeur de logP[9], sans pour autant qu'une règle claire ne se dégage. En effet, la longueur de chaine cinétique de la synthèse enzymatique d'un polyester ne dépend pas uniquement de l'affinité de l'enzyme pour le substrat, mais également de la solubilité du monomère et du polymère et des coproduits éventuels[13] d'autant que cette solubilité va par ailleurs varier avec le poids moléculaire du polymère formé).

L'immobilisation de l'enzyme a également fait l'objet de très nombreuses études. Toutefois, si l'intérêt de l'immobilisation a clairement été démontré, parmi les différentes méthodes d'immobilisation (sorption[14], liaison covalente[15]) ou les différents substrats (argiles[16], polymères[14], verre ou céramique[17]) aucun ne semble donner des résultats vraiment supérieur aux autres en ce qui concerne la eROP de lactones.

Les paramètres de structure des monomères sont également extrêmement importants pour les réactions enzymatiques. Dans la nature chaque enzyme fonctionne généralement pour un nombre limité de substrats et cette sélectivité peut-être aussi bien un avantage qu'un handicap pour son utilisation dans d'autres réactions. Ainsi, deux monomères très proches pourront être polymérisés sélectivement par deux enzymes distinctes comme c'est le cas, par exemple du L- et du D-lactide, respectivement polymerisés par la lipase de *Burkholderia cepacia*[18] et par la lipase B de *Candida antarctica*[19,20].

Objet de l'étude

L'objet de cette étude consiste à déterminer l'influence de la structure des monomères sur les polymères obtenus par ouverture de cycle et notamment à analyser l'influence de la taille du cycle, de sa stéréochimie, de la nature de l'hétéroatome cyclique et de la présence d'éventuels substituants.

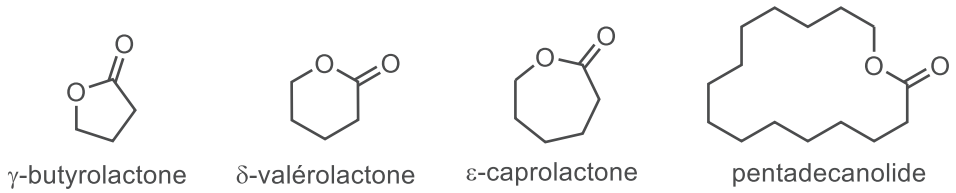


***Figure I-4**. Exemples de lactones de différentes tailles de cycles*

Tout d'abord, concernant l'influence de la taille du cycle, de nombreuses lactones ont été polymérisées par diverses lipases, produisant des polymères et des oligomères de taille très variable allant d'un degré de polymérisation de quelques unités à une centaine d'unités. Une tendance forte semble toutefois se dessiner dans la littérature. En effet, si la ROP chimique des lactones semble fortement contrôlée par l'enthalpie. Donc, les monomères à forte contrainte de cycle tels que le lactide et la β-propiolactone sont polymérisés beaucoup plus facilement que les macrolactones, la ROP enzymatique quant à elle semble être plus efficace pour les monomères à faible contrainte de cycle. Plus celle-ci est faible, plus le degré de polymérisation semble élevé Il existe cependant dans la littérature quelques résultats atypiques en ROP enzymatique rapportant l'obtention de polyesters de haute masse molaire à partir de monomères ayant une contrainte de cycle importante[21–23].

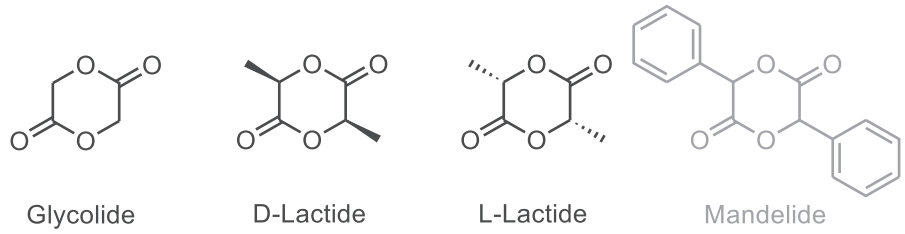
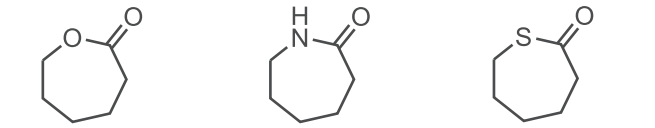


***Figure I-5**. Examples de dilactone avec variation du substituant ou de sa stéréochimie*

Les substituant des lactones semblent également avoir une grande influence sur la polymérisation. En effet, l'encombrement de certains groupements latéraux peut empêcher le monomère de s'insérer correctement dans le site actif de l'enzyme, et pour cela chaque lipase a ses spécificités. La lipase B de *Candida antarctica* a ainsi montré une plus grande efficacité en l'absence de substituants sur le cycle comme par exemple pour la δ-valérolactone ou encore le glycolide. Mais dans le cas où un substituant est présent cette même lipase a montré une sélectivité pour les substrats (S) tel que le D-lactide, à l'inverse de

la lipase de *Burkholderia cepacia* qui a montré une certaine sélectivité pour les substrats (R) tel que le L-lactide[18].

Enfin, un troisième paramètre relatif au monomère a été assez peu étudié, il s'agit de l'influence de l'hétéroatome intra-cyclique. D'un point de vue chimique, les atomes d'oxygène, de soufre, de sélénium et d'azote ont généralement des réactivités comparables et il est possible chimiquement de les faire réagir dans des conditions très proches. Par exemple, la caprolactone et ses équivalents azotés[24] peuvent être polymérisés par des dérivés d'étain et également par voie ionique tout comme leurs équivalents soufrés[25] .



ε-caprolactone ε-caprolactame ε-thiocaprolactone

Figure I-6. *hétérocyclocarbonyl à 7 chainons*

Ce troisième paramètre a fait l'objet de bien moins d'études que ceux précédemment évoqués. Toutefois, certains auteurs ont montré qu'il était possible, dans une certaine mesure de polymériser ou de copolymériser des équivalents soufrés[26] et azotés[27] des lactones.

Organisation du manuscrit

En amont de la conclusion générale, ce manuscrit est structuré en 4 grand chapitres organisés autour de diffèrents articles scientifiques rédigés en anglais et encadré par des textes d'introduction et de conclusion en français.

Dans le **chapitre un**, rédigé en anglais, des généralités sur la polymérisation enzymatique et plus spécifiquement sur la polymérisation par ouverture de cycle enzymatique seront présentés. Dans ce chapitre une attention toute particulière sera portée sur l'influence de la structure des monomères sur l'activité catalytique des lipases, que ce soit la taille et la contrainte des cycles, les substituants et leur stéréochimie ou la nature des hétéroatomes présents.

Dans un **second chapitre**, nous étudierons précisément d'un point de vue expérimental la polymérisation enzymatique de lactones, l'influence des substituants et de leur stéréochimie et également l'influence de la contrainte de cycle sur la structure des architectures macromoléculaires obtenues, ainsi que l'influence de certains solvants.

Le **troisième chapitre** présente la polymérisation de caprolactone et de son équivalent soufré, la thiocaprolactone (thiepan-2-one). L'influence de la substitution de l'oxygène intra-cyclique par le soufre y est plus particulièrement discutée.

Le **quatrième chapitre** analyse et discute l'influence de l'azote, en tant qu'hétéroatome. Tout d'abord via l'eROP de caprolactame (équivalent azoté de la caprolactone) et d'anhydride d'acide aminé. Puis les résultats de copolymérisations de la caprolactone avec deux acides aminés distincts seront présentés.

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II. A brief review on enzymatic catalysis for the ring opening polymerization (ROP) of lactones

Introduction

Enzymes are the catalysts of the natural world. In each biosynthesis, the final compound is obtained after a cascade of reactions, and each step is catalyzed by, at least, one enzyme. Above all, these reactions are conducted under biological conditions, in aqueous environment and at low temperature. The incredible diversity and complexity of molecules that can be produced this way show the versatility and efficiency of these natural catalysts, and make them extremely attractive for the synthetic chemist.

Enzymes are proteins, i.e. polymers composed of amino-acids, and their tridimensional structure is one of the most important parameters that make the specificity and the catalytic activity of each enzyme. It is this structure that allows or not the enzyme binding with a given substrate thus, any modification of this 3D conformation can significantly modify the enzyme catalytic activity.

Enzymes are classified in 6 major classes; depending on the type of reaction they catalyze (Figure 1).

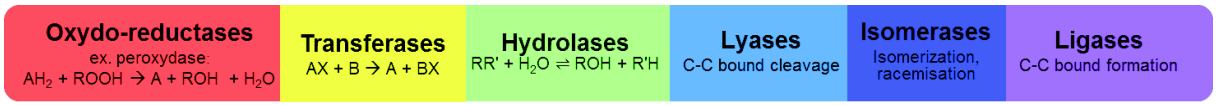

Figure II-1. *The main enzymes classes and the type of reaction they catalyze*

Some enzymes intervene in the biosynthesis of polymers that are constitutive of the biological world, such as polysaccharides (cellulose, starch, chitin, glycogen…), proteins or polyphenols (lignin, tannins…). Other less common biosynthesized polymers include

polyhydroxyalkanoates (PHA), which are polyesters produced by some bacteria as energy storage under environmental stress[1] The field of polymer synthesis concerned by enzymatic catalysis is thus really wide, and includes numerous different types of chemical reactions[2]

The whole idea of enzymatic catalysis in polymer science is to use the natural ability of enzymes to catalyze the formation of biopolymers, and to exploit it to obtain synthetic polymers. Different kinds of artificial polymers have been obtained through enzymatic catalysis, such as polyamides[3], but the most widely studied are undoubtedly polyesters[4,5]

Polyesters can be obtained by two different types of reactions, polycondensation or ring opening polymerization (ROP) (Figure 2). The latter presents some advantages: a direct and precise control of the stoichiometry, an easier reaction control due to the absence of side products, and energy efficiency because of the release of ring strain during the reaction (enthalpy-driven reaction).

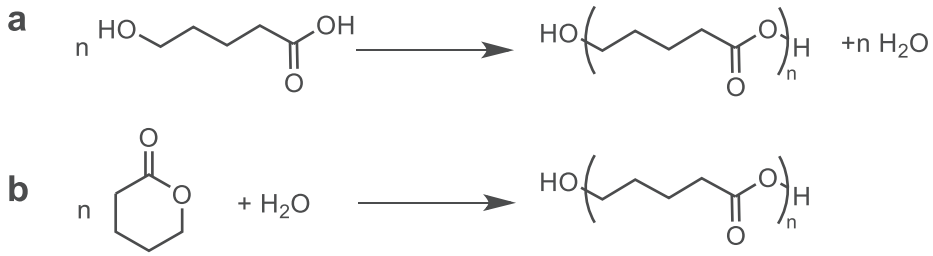

Figure II-2. *Examples of polyester synthesis by polycondensation (a) or ROP (b)*

Today, most of the industrially produced polyesters are obtained using organometallic catalysts. Even though the technique is efficient and well-mastered, it presents several drawbacks, like the catalysts toxicity for humans and the environment[6,7] As a cleaner, greener and safer alternative, enzymatic catalysis for the synthesis of polyesters has known an increasing interest in the past decades, especially concerning the ROP of lactones and their derivatives[8]

Figure 3 shows the linear increase in the number of publications on enzymatic polymerization over the last 30 years. Nowadays, with more than 300 publications each year, the general comprehension of the mechanisms, as well as the development of potential applications, evolve very quickly. If enzymatic polymerization could be considered

as a lab curiosity 30 years ago, it is no longer the case today, and intensive researches are conducted to make the method competitive in large-scale production.

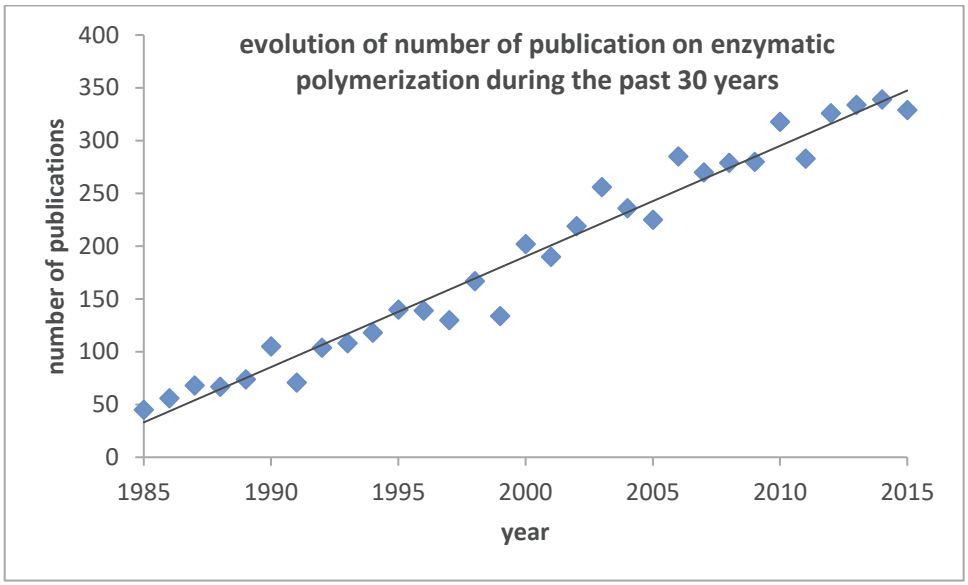

Figure II-3. *Evolution of the number of publication on enzymatic polymerization over the past 30 years (data from SciFinder scholar collected in January 2016; excluding patents, review articles and book chapters).*

This brief literature overview focuses on the enzymatic catalysis for the ROP of lactones. It aims at showing the different kind of enzymes that have been used, and at evidencing the key parameters that can be tuned to improve the polymerization.

Enzyme-catalyzed ROP (eROP) of lactones

Chemically, the ROP of lactones is a specific form of transesterification. It thus seems logical that esterases (E.C. 3.1), a group of enzymes that are able to synthesize ester functions, were the first choice to attempt to synthesize polyesters. Figure 4 presents various esterases that were studied and showed an activity for polyester synthesis.

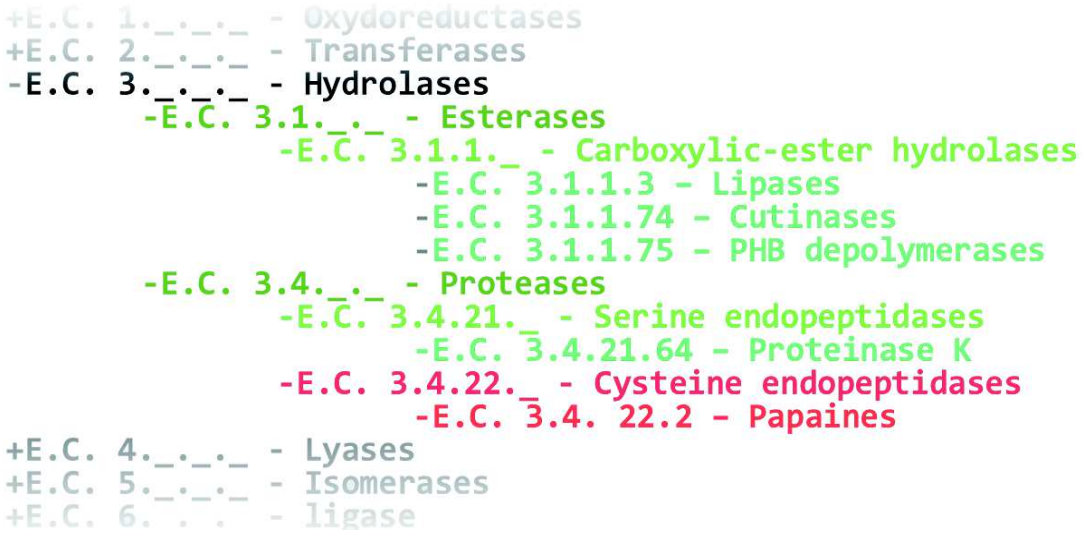

Figure II-4. *Enzymes types that have shown an activity in enzymatic synthesis of polyesters*

First, the enzymes that are involved in the natural synthesis or hydrolysis of polyesters like PHA[9] were considered. Various studies in the literature report that some PHA-depolymerases (E.C. 3.1.1.75-76) have promising activity in the eROP of lactones, especially on short or medium ring size lactones (4 to 7 ring members)[10–13]. PHA-depolymerases are serine hydrolase (hydrolase which is bound on a serine residue) but these enzymes are not very well known: most of them are not yet perfectly characterized and only very few of them are commercial. This explains why there are only few studies on their use in eROP until now.

Lipases (E.C. 3.1.1.3) are the most studied enzymes for ROP (Table 1). They can be found in most organisms from the microbial, vegetal and animal kingdom[14]. Like PHA-depolymerases, they are serine hydrolases that catalyze ester bond cleavage in aqueous medium (their physiological action is the cleavage of triglyceride), but they are also able to catalyze ester bond formation in organic medium (the reverse reaction)[8].

More recently another esterase, cutinase (E.C. 3.1.1.74), has been tested with very promising results on a wide range of lactones from 6-membered lactones to 16-membered macrolides[15,16]. Cutinases can be found, among other, in plant pathogenic fungi. Like the two other esterases previously cited, they are also serine hydrolases that catalyze, when in their natural environment, the hydrolysis of cutin, a waxy polyester of ω-hydroxy fatty acids. Some esterases form mammalian liver (rabbit and hog) have also been tested for eROP, but they did not lead to significant monomer ring opening[17,18].

Interestingly, protease (E.C. 3.4.21), another family of enzymes present in every living organism has been tested in the eROP of lactone, but produced mixed results so far[17,19,20]. Enzymes of this class naturally cleave amide bonds in peptides and proteins. Considering the chemical similarity between ester and amide functions, it is not that surprising that these enzymes can be able to open lactone rings. It is interesting to notice that all proteases that have shown an activity in ROP are, like all previously cited enzymes, serine hydrolases. However, it is noteworthy hat another protease, papain, which is a cysteine hydrolase (E.C. 3.4.22.) instead of a serine hydrolase, has also shown some activity on ester functions (for hydrolysis and interesterification)[21], but has not been yet tested for eROP catalysis.

Table 1 summarizes all enzymes that have shown an activity in eROP catalysis. It is interesting to notice that the tested enzymes only represent a very few number of the numerous known serine hydrolases.

Table II-1. List of enzymes reported as active in eROP of lactones, sorted according the enzyme type and lactone ring size, with corresponding references.

organism	enzyme	enzyme modification	ring size [a]	References
Alcaligenes faecalis	Lipase		large	22
Alcaligenes faecalis	PHB depolymerase		short	11,12
			medium	11
Aspergillus niger	Lipase		short	23
			medium	24–26
			large	18,24,27
Bacillus thermoproteolyticus rokko	protease		medium	17

Table II-1 . **continuation**, enzymes that have been reported as active in ROP of lactones in function enzyme type and of ring size

organism	enzyme	enzyme modification	ring size [a]	References
Burkholderia cepacia	Lipase		short	23,28–31
			medium	17,19,20,24–26,28,32–44
			large	18,24,27,42,45–49
Burkholderia cepacia	Lipase	immobilized	short	50
Burkholderia cepacia	Lipase	surfactant coated	medium	45
Candida antarctica	Lipase A		short	51
Candida antarctica	Lipase B		short	20,23,51
			medium	34,35,52–54
			large	24,46,47,52,54,55
Candida antarctica	Lipase B	immobilized	short	13,30,31,50,51
			medium	13,16,17,26,33,36,38–41,44,49,56–77
			large	16,49,78–84
Candida antarctica	Lipase B	immobilized, Ionic liquid coated	medium	75,85
Candida cylindracea	Lipase		short	29–31,86
			medium	18–20,22,24,25,31,33,34,37,87,88
			large	18,22,24,27,37,46,48
Candida rugosa	Lipase		short	51,89,90
			medium	17,26,38–40,62,91
			large	18,27
Candida rugosa	Lipase	immobilized	medium	62
Humicola insolens	Cutinase		short	16
			medium	15,16
			large	15,16
Mucor javanicus	Lipase		short	30
Mucor javanicus	Lipase	immobilized	short	30
Penicillium roqueforti	Lipase		large	18

Table II-1. continuation, enzymes that have been reported as active in ROP of lactones in function enzyme type and of ring size

organism	enzyme	enzyme modification	ring size a	References
porcine pancreas	Lipase		short	28–31,86,89,90
			medium	17,20,24–26,28,31,33,34,36–41,87,88,91–95
			large	18,22,27,34,47,48,96
porcine pancreas	Lipase	immobilized	medium	88,95,97,98
Proteinase K	protease		medium	19,20
Pseudomonas aeruginosa	Lipase		short	23
			medium	24
			large	24,46
Pseudomonas fluorescens	Lipase		short	23,99
			medium	18,22,24–26,33,34,37,42,52,62,87,100–102
			large	18,22,24,27,37,42,46–48,52,54,102,103
Pseudomonas fluorescens	PHB depolymerase		short	13
			medium	13
			large	13
Pseudomonas sp.	Lipase		short	90,104,105
			medium	36,38,104,105
			large	22,84,104
Pseudomonas stutzeri	PHB depolymerase		short	10
Rhizomucor miehei	Lipase		medium	17,26,33,34,62
			large	54
Rhizomucor miehei	Lipase	immobilized	medium	88,94
			large	84
Rhizopus japonicus	Lipase		medium	25,37
Rhizopus oryzae	Lipase		medium	17,25
Thermomyces lanuginosus	lipase		short	51
Thermophilic ESL-001 series	Lipase		short	106
Yarrowia lipolytica	lipase		medium	107,108

[a] *short ring size corresponds to monomers with 3 to 5 ring members, medium corresponds to 6 to 8 ring membesr, and large corresponds to lactones with more than 8 ring members.*

Enzymes tridimensional structures

The enzyme activity is closely related to its tridimensional structure. The structure should be well adapted to the natural substrates of the enzymes. In the case of lipases or cutinases, the enzyme surface should present large apolar area to allow the formation of an acyl-enzyme complex with fatty acids or fatty esters. Interestingly, some lipases have the capacity to change their structure, *i.e.* to switch from an open to a closed state of the binding site[109–111]. Most important results on the enzymes structure were obtained by Pleiss and coworkers[112], who showed that lipases could be classified into 3 major groups depending on the form their binding site (Figure 5). Lipases can present either a tunnel-shape binding site, like for *Candida rugosa*, a crevice-like binding site like for *Rhizomucor miehei*, or a funnel-shaped binding site like for lipases from *Candida antarctica* and lipase from *Burkholderia cepacia.* The latter ones, with a funnel-like binding site, are those presenting the highest efficiency.

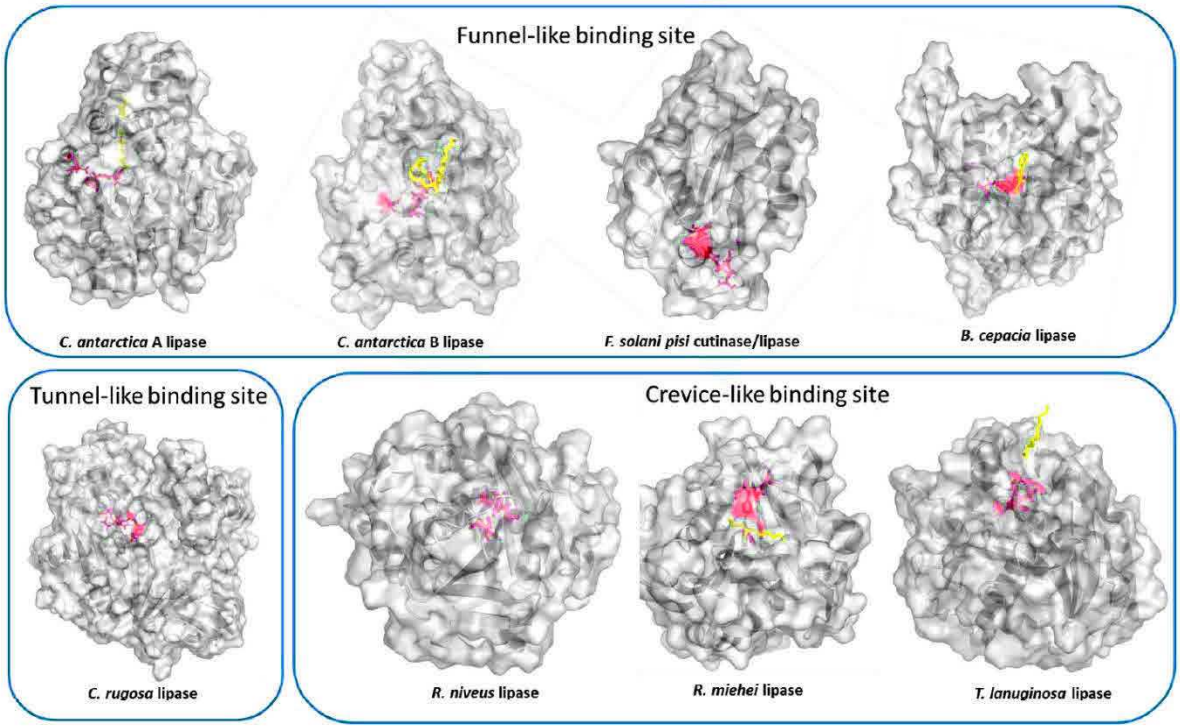

Figure II-5. *Lipases binding sites structures, reproduced with authorization from Pleiss et al*[112]

Most of the enzymes have shown some selectivity, chemoselectivity or stereoselectivity, which are also closely related to the structure of their binding sites[113]. Regarding

chemoselectivity, lipases seem to be more efficient on lactones ω-substituted with long alkyl chains, which likely have the ability to stabilize the acyl-enzyme complex by apolar interactions between the enzyme and the monomer[58,63]. On the contrary, α-substituted lactone show very poor activity in eROP[54]. Stereoselectivity has been reported for example on lipases from *Burkholderia cepacia*, which show some selectivity for L stereoisomers[113], whereas lipase B from *Candida antarctica* shows a great selectivity toward D stereoisomers[114–116].

Serine hydrolase mechanism pathway

As previously stated, all enzymes presented in Table 1 are serine hydrolases. This means that they all have the same catalytic mechanism, involving a catalytic triad composed of a serine (Ser), a histidine (His) and a glutamate or an aspartate (Glu/Asp). The esterification mechanism catalyzed by serine hydrolase has been well described[117]. The activity is based on an activation of serine alcohol by hydrogen bonding assistance of His and Glu/Asp moities, as described on Figure 5.

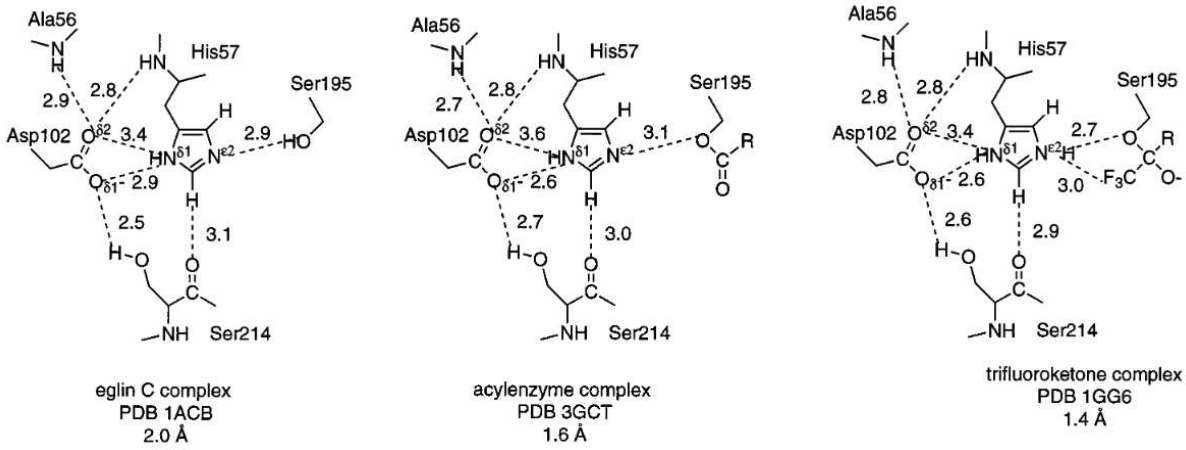

Figure II-6. *Serine alcohol activation by hydrogen bonding in catalytic triad of chymotrypsin, reproduced with authorization[117]*

The application of this catalytic mechanism to lactone ring opening polymerization has been proposed by Kobayashi in 2006, and is reproduced on Figure 6[118],

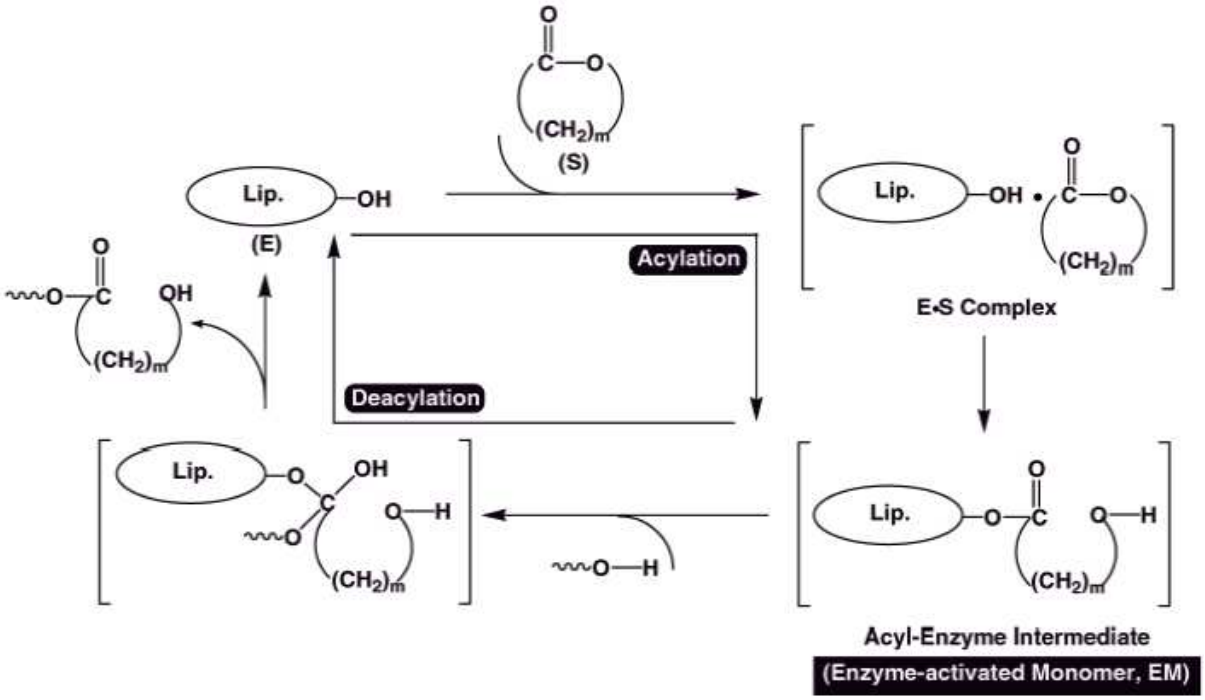

Figure II-7. *Catalytic mechanism of eROP proposed by Kobayashi in 2006, reproduced with authorization*[118]

Like many polymerization reactions, the eROP reaction can be divided in 3 major steps: initiation, propagation and ending. The initiation step, presented on Figure 7, consists in an acylation stage, with the formation of an acyl-enzyme complex, followed by the nucleophilic attack of water[96] or another initiating alcohol[73,81,92,103]. The acyl-enzyme complex then releases a mono-adduct close to the enzyme binding site. This mono-adduct can later be involved in a new acylation-deacylation cycle as nucleophilic agent, and so on until the complete consumption of the monomers.

The kinetic chain resulting of this mechanism has been described by Gross and coworkers[78,119], and showed very good correlation with experimental data reported by Johnson et al[120]. The resulting kinetic pathway is summarized in Figure 8[120].

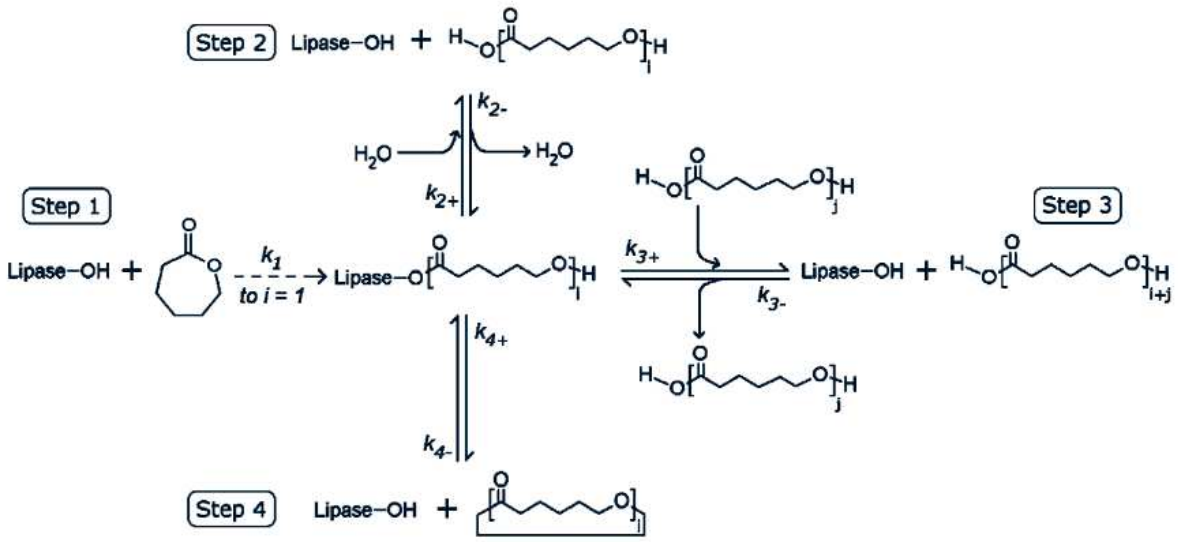

Figure II-8. *Model kinetic pathway of eROP of ε-caprolactone, reproduced from Johnson et al. with authorizations*[120]

Regarding this kinetic pathway, it seems obvious that the water content is a key parameter of the kinetic chain length. According to this pathway, water should be totally removed to increase the final polymer chain length. However, the enzymes activity is correlated with their water content. Indeed, the tridimensional structure of enzymes is strongly depending on their water content. To maximize the molar mass without reducing the conversion or increasing dramatically the reaction time, an optimal water content has to be found.

Despite numerous studies, an ideal water content could not be found[59,92,96,105]. Dong et al[105] showed that the reaction rate increased with an increase in water content, but caused a lowering of the molar mass. Surprisingly, Mei and coworkers did not observe any modification of the reaction rate with the increase in water content. They explained this fact by a limitation of the reaction rate by monomer activation[59]. Other works tried to maximize enzymes activity by varying the solvent log P, but did not manage to evidence an influence of log P on eROP of lactones[25,105].

Enzymes modifications

To increase their activity or facilitate their handling, enzymes can be modified. The most common modification of enzymes is immobilization. In fact, most of eROP studies have been done with immobilized enzymes, such as Novozyme 435 (lipase B from *Candida antarctica* immobilized on acrylic resin), which is the most widely commercially available lipase. Immobilization allows, *inter alia*, a better thermal stability and an easier recovery of the catalyst. In parallel to such porous acrylic resin beads, various alternative enzyme supports like porous ceramic (silica) or other polymer based carriers have also been investigated[8]. Recently, some interesting enzyme immobilizations on nanoclays were reported by our group[77,121]. These supports could provide interesting perspectives for modifying the final materials properties and be use as reactive filler. Enzymes modification by immobilization have been extensively described[2,122], and will thus not be detailed here.

Another way to improve the enzyme activity for eROP is the coating with compounds that are able to facilitate the reaction. Two types of coating have been reported. Surfactant coating helps to improve the dispersion of enzymes in an organic solvent, and minimize the exchange of residual water of the enzymes with the reactive medium[45]. Ionic liquid coating aims at improving the miscibility of the enzymes with the monomers, but did not lead to significant improvements[75].

eROP of lactones: main results reported in the literature

This part will focus on eROP results and will discuss the main parameters that influence this reaction and will also list the main improvement ways that have been tested. Table 2 summarizes the main results on eROP of simple lactones as a function of their ring size.

Table II-2. Results from the polymerization of various simple lactones by different enzymes

monomer	solvent / system	T (°C)	t (h)	Mn (g.mol^{-1})	ξ[a]-η[b] (%)	references
		Pseudomonas fluorescens PHB depolymerase				
β-propiolactone	bulk	80	6	9 200	100[a]	13
γ-butyrolactone	bulk	80	24		0[a]	13
ε-caprolactone	bulk	80	48		0[a]	13
ω-pentadecanolide	bulk	80	48		98[a]	13
		Alcaligenes faecalis PHB depolymerase				
β-propiolactone	bulk	60	48	10 800	98[a]	11
γ-butyrolactone	bulk	80	48	0		11
δ-valerolactone	bulk	80	48	3 200	92[a]	11
ε-caprolactone	bulk	80	48	350	32[a]	11
		Aspergillus niger Lipase				
β-propiolactone	bulk	60	120	850	32[a]	23
ε-caprolactone	bulk	60	240	780	4[b]	24,25
ω-dodecanolide	bulk	60	120	1 200	12[b]	24,27
ω-pentadecanolide	bulk	60	240	2 800	16[b]	18
		Burkholderia cepacia Lipase				
β-propiolactone	bulk	60	120	300	60[b]	23
γ-butyrolactone	hexane	60	430	900	42[b]	28
ε-caprolactone	bulk	60	240	6 100	84[a]	25, 37
ω-octalactone	isooctane	60-75	240	9 700-16 000	81-85[a]	24, 46
ω-undecanolide	cycloexane	60	72	12 600	91[a]	45
ω-dodecanolide	bulk	60	120	5 600	100[a]	27, 24
ω-pentadecanolide	bulk	60	240	3 500	12[a]	18
		Candida antarctica Lipase B				
β-propiolactone	bulk	60	120	500	71[b]	23
δ-valerolactone	toluene	45	8	19 000		80
ε-caprolactone	scCO2	35-65	2-72	18 000-54 000	69-98[a]	61
ε-caprolactone	toluene	70	24	29 400		16
ω-heptalactone	toluene	45	8	23 600		80
ω-octalactone	toluene	45	8	20 700		80
ω-nonalactone	toluene	45	8	16 000		80
ω-decanolide	toluene	45	8	20 000		80
ω-undecanolide	toluene	60	24	28 000	98[a]	54
ω-tetradecanolide	toluene	60	24	13 000	94[a]	54
ω-pentadecanolide	toluene	70	2	49 000-86 000	77-90[b]	78, 79, 82

Table II-2. continuation, Results from the polymerization of various simple lactones by different *enzymes*

monomer	solvent / system	T (°C)	t (h)	Mn (g.mol^{-1})	ξ^a-η^b (%)	references
			Candida cylindracea Lipase			
β-propiolactone	bulk	60	48	49100	99[a]	86
δ-valerolactone	bulk	60	120	1400	77[a]	24, 25
ε-caprolactone	bulk	75	240	25200	95[a]	18
ω-octalactone	isooctane	60	240	500	15[a]	46
ω-undecanolide	bulk	75	240	21 000-25 200	95[a]	24, 37, 22
ω-dodecanolide	bulk	75	120	13 000	99[a]	24, 37
ω-pentadecanolide	bulk	75	240	16 200	65[a]	18, 24, 37
ω-hexadecanolide	bulk	75	120	5 800	100[a]	37
			Humicola insolens Cutinase			
γ-butyrolactone	toluene	70	24		0[a]	16
ε-caprolactone	toluene	70	24	24 900	99[a]	15, 16
ω-pentadecanolide	toluene	70	24	44 600		16
			porcine pancreas Lipase			
β-propiolactone	bulk	60	48	36 500	96[a]	86
γ-butyrolactone	hexane	60	430	900	25[b]	28
δ-valerolactone	bulk	60	120	1 100	96[a]	34, 25
ε-caprolactone	bulk	60	240	2 500	69[a]	87
ε-caprolactone	heptane	65	96	1 600-2 700	100[a]	92
ω-undecanolide	bulk	60	120	5 800	85[a]	22
ω-dodecanolide	bulk	60	120	3 200	99[a]	27, 24
ω-pentadecanolide	bulk	60	240	1 800	27[a]	18
			Pseudomonas aeruginosa Lipase			
β-propiolactone	bulk	60	120	650	26[b]	34
ε-caprolactone	bulk	60	240	2 700	56[a]	24
ω-dodecanolide	bulk	60	120	7 700	99[a]	24
			Rhizomucor miehei Lipase			
ε-caprolactone	diisopropyl ether	65	5		45[a]	62
ω-undecanolide	toluene	60	24	3 200	40[a]	54
ω-tetradecanolide	toluene	60	24	5 700	75[a]	54
ω-pentadecanolide	bulk	80	72	15 000-34 000	92[a]	84
			Pseudomonas fluorescens Lipase			
β-propiolactone	bulk	60	120	650	84[b]	23
δ-valerolactone	bulk	60	240	1 700-2 100	91[a]	87, 100, 24, 25
ε-caprolactone	bulk	60-75	240	8 300-22 800	99-100[a]	18, 24
ω-octalactone	isooctane	60	240	8 500	38[a]	24, 46
ω-undecanolide	bulk	75	240	22 800	100[a]	24, 37, 22
ω-dodecanolide	bulk	75	120	9 600	100[a]	37
ω-tetradecanolide	toluene	60	24	5 200	40[a]	54
ω-pentadecanolide	bulk	60-75	240	2 800-7 500	97-99[a]	18, 24, 37, 22
ω-hexadecanolide	bulk	60	120	2 600	99[a]	37

[a] *monomer conversion,* [b] *polymer yield*

Influence of the ring size

Very few enzymes have been reported as active in the ROP of γ-butyrolactone, the most simple 5-membered lactone ring. Only limited conversions were reached: 25% and 42 % with lipase from porcine pancreas or from *Burkholderia cepacia*, respectively[28]. Furthermore, the final molar mass was low (about 900 g mol^{-1}, corresponding to 10 monomers units) and the reaction time extremely long (up to 18 days)[28]. This limited activity could be explained by the poor affinity between short polar compounds and the lipase binding site, which is known to present a large apolar area. The good chemical stability of 5-membered lactones could also be an additional limiting factor.

A close examination of data presented in Table 2 leads to some interesting information on the influence of the lactone ring size. Enzymes could be tentatively classified depending on the evolution of their efficiency with the lactone ring size.

First, lipases from *Aspergillus niger, Rhizomucor miehei, Pseudomonas aeruginosa* and cutinases from *Humicola insolens* show an efficiency that increases with the ring size. Even though this pattern seems clear, it should be tempered because of the limited quantity of studies and data on these enzymes.

Then, PHB depolymerases follow the opposite trend. They seem to be more efficient on short rings, the greater activities being reported on four-membered lactones: on β-butyrolactone for depolymerases from *Pseudomonas stutzeri*[10], or on β-propiolactone for depolymerases from *Pseudomonas fluorescens*[13] and *Alcaligenes faecalis*[11] This last enzyme has also shown a limited activity on larger lactones, but only with a low degree of polymerization (oligomers of 3 monomeric units for ε-caprolactone[11]). Such behavior was expected, since this class of enzymes is designed to hydrolyze PHA, which are mostly composed of short hydroxyacids units (hydroxyvalerate and/or hydroxybutyrate units).

Finally, a third group of enzymes does not show any clear evolution of efficiency with the ring size. It includes lipase B from *Candida antarctica,* and lipases from porcine pancreas, *Candida cylindracea, Burkholderia cepacia* and *Pseudomonas fluorescens.* Each of these lipases shows a maximum in efficiency for a specific lactone ring size. Lipase from *Burkholderia cepacia* has a maximum activity for 9-membered lactone (ω-octalactone),

lipase from porcine pancreas for 12-membered ω-undecanolide, lipase B from *Candida antarctica* for 16-membered ω-pentadecanolide, and lipases from *Candida cylindracea* and *Pseudomonas fluorescens* for 7 to 12-membered lactones (from ε-caprolactone to ω-undecanolide). The difficulty in evidencing a clear trend for these enzymes also comes from the numerous studies and large diversity of data, coming from many different research teams.

Even if a clear trend can hardly be seen for this last group, it generally seems that lipases are more active on large lactones, whereas PHB depolymerases are more efficient on shorter rings. This behavior can be rationalized by the similarities with the natural substrate of these enzymes: short ring lactones are closer to the natural hydroxyacids hydrolyzed by PHB depolymerases, whereas large lactones are closer to the fatty acids that are usually hydrolyzed by lipases.

Influence of the reaction conditions

The results presented in Table 2 show that each enzyme seems to have its own specific optimal conditions to maximize its efficiency in eROP. Some enzymes work better in bulk conditions, such as PHB depolymerases and lipases from *Pseudomonas fluorescens, Pseudomonas aeruginosa,* porcine pancreas, *Candida cylindracea,* and *Aspergillus niger*. On the other hand, lipases from *Candida antarctica* and *Rhizomucor miehei* and cutinase from *Humicola insolens* seem to be more efficient in apolar organic solvents, such as toluene or isooctane. Interestingly, lipase from *Burkholderia cepacia* appears to be efficient in both solvent and bulk conditions.

Lipases and cutinases usually work at the interface between aqueous and organic phases, because their natural substrates are triglycerides and hydroxy-fatty acids. To mimic the natural working conditions of the enzymes, some authors tried to perform eROP in mini-emulsion or in water dispersion, but the results were not satisfying. If there is a drastic decrease in the reaction time to reach complete monomer conversion (from a few days to a couple of hours), the obtained oligomers were much too small to be useful for most of applications (only 2 to 5 monomer units per chain)[32,42].

In the past few years, ionic liquids have been intensely investigated as an alternative to classical organic solvent[67,123], especially by Mena and coworkers[69,70,73]. However, none of the tested conditions have yet exceeded the results obtained in classical conditions.

The use of supercritical fluids, especially $scCO_2$, is another way to substitute organic solvents[43,61,68,74,124,125]. It has been widely explored as a promising green method, allowing the reduction of the reaction temperature, and in some cases also of the reaction time[61]. Two other supercritical fluids have been used in eROP, liquefied petroleum[125] and supercritical tetrafluoroethane (scR-134a)[43]. In the latter case, the use of scR-134a as solvent for the eROP of lactide catalyzed by lipases from *Burkholderia cepacia* led to greater molar mass than the classical conditions (up to 14 000 g mol^{-1}), but the polymer yield did not exceed 50%, and the reaction time and temperature were not reduced. Moreover, the use of scR-134a instead of toluene does not seem such a better choice from a green chemistry perspective.

All the tested enzymes have given best results around 60°C. But this has to be taken with caution, because in many cases there are too little data in the literature to be categorical on a possible optimal reaction temperature.

Possible strategies for eROP improvement

Besides the substitution of organic solvents by supercritical fluids or ionic liquids, some other less conventional methods have been tested to improve eROP efficiency, including microfluidic processes[72,126], microwave irradiation[66], ultrasound activation[51,71,127] or reactive extrusion[83].

Microwave irradiation has been tested for the ROP of caprolactone catalyzed by Lipase B from *Candida antarctica*[66], but it did not lead to significant improvements. It slightly increased the reaction rate in diethyl ether, but decreased it in better solvents for the enzyme such as toluene or benzene.

Ultrasound activation also leads to significant improvement of the eROP, with final polymers of molar mass three times greater than in classical conditions. It can be done once at the

beginning of the reaction (few minutes at high power)[71], or during the whole reaction at lower power[51]. The use of aontinuous flow reaction, such as in microfluidic processes or reactive extrusion, is also a very promising way of improvement. Microfluidic processes have demonstrated a spectacular improvement of reaction kinetics. Bhangale and coworkers[126] have performed eROP with almost quantitative ε-caprolactone conversion in 120 s instead of a least 8 h without significant molar mass variation of the final PCL. More recently, reactive extrusion has been tested for the eROP of ω-pentadecanolide with immobilized lipase B from *Candida antarctica*[83]. Here again, results are spectacular, with an almost total monomer conversion in only 15 min, resulting in 80 000 g mol^{-1} polymers. To obtain similar results in classical batch conditions, 72 h would be necessary. In addition to accelerate the reactions kinetics, this last process also has a great potential for future large scale polymerization processes.

Other heteroatoms in cyclic monomers: lactams and thiolactones

Proteases, enzymes whose natural function is amide linkage hydrolysis, could be efficient on ester bonds formation. Similarly, lipases or cutinases, enzymes whose natural function is ester linkages hydrolysis, could also be able to form amide bonds.

Enzymatic synthesis of polyamides[128] by ROP is much more difficult than polyesters synthesis, and only few works were performed on the eROP of lactams. These studies present interesting results, but most of the time polymer chains with only a few lactam units were obtained[129]. Besides, according to some simulation works, it seems difficult to obtain long polyamides chains, because of the great stability of large lactams compared to β-lactam[130]. Lipases were shown to catalyze the ROP of lactams from five-membered cycle to nine-membered cycle, as well as the formation of cyclic amino acids dimers and trimers[131]. Some other strategies have been used to synthesize polyamides or polyesteramides, such as polycondensation reactions catalyzed by lipases (synthesis of low molar mass poly(aspartic acid) for example[132]), or eROP catalyzed by protease instead of lipases or cutinases which are the most commonly used enzymes[132–135]. Some authors also tried to modify enzymes by mutagenesis, aiming at improving polyamide production, but with mixed results so far[136].

If lactones are chemically close to lactams, they are even closer to thiolactones, their sulfur equivalent. Enzymatic transthioesterification has been used in several organic synthesis reactions[137–141], for waxes synthesis for example[142], but eROP of cyclic thioesters has not been widely studied so far. Matsumura et al. proved that this kind of function can be polymerized by lipase with promising results[143,144]. Other authors performed eROP of thiolactones, such as Wu et al. with beta-thiolactone[141] or Weber et al. with macrothiolactone[145]. Unfortunately, there are very few works on thiolactones eROP[138,140,142,146,147]. In their work, Matsumura and coworkers report some interesting thermal properties for poly(ester-co-thioester) that could be tuned by the variation of the thioether linkage content of copolymers[138]. Hedfors et al. also reported a better reactivity of thiol functions than ester functions, and stated that N435[148] seems to have short kinetic chain length and is only able to produce oligomers. All these works are promising but there are still many unknown parameters and untested possibilities, thus requiring further investigations.

Conclusions

All the results reported here tend to confirm the hypothesis that ring strain is not the main driving force in lactones enzymatic ROP. Indeed, large ring lactones, such as ω-pentadecanolactone, ω-dodecanolactone or ω-decanolactone, for which the ring strain can be considered as weak or null, are also those showing the quickest reactions and reaching the highest molar masses.

However, lactones having the highest ring strain, such as β-propiolactone or *O*-carboxyanhydride of lactide, are not those exhibiting the lowest reactivity. The 5-membered monolactone, γ-butyrolactone, is indeed the one having the lowest reactivity This is in accordance with general organic chemistry behavior that generally shows a great stability for 5- and 6-membered cycles. These results lead to hypothesize that, if the ring strain is an important parameter, it is necessarily in balance with another which could be the stability of the acyl-enzyme complex. For lactones smaller than 5-membered cycles, the ring opening seems to be sufficiently fast to drive the reaction before the acyl-enzyme complex breaks,

whereas for very large cycles without ring strain, the acyl-enzyme complex is probably very stable due to the chemical similarity with the lipases natural substrates (fatty acids).

This hypothesis could explain why γ-butyrolactone could not be directly polymerized by eROP. The acyl-enzyme complex is probably not very stable because of the high polarity of the 5-membered lactone which is not compatible with the large apolar area of the lipases binding site. Furthermore, 5-membered lactone rings are quite stable under classical ROP conditions.

The kinetic chain length also seems correlated to the ring size. As for reactivity, larger lactones exhibit greater degree of polymerization (easily up to 400 units for ω-pentadecanolactone polymers, while β-propiolactone exhibits most of the time a DP lower than 30). These data lead us to think that, as there is a monomer-enzyme complex, there is also a "growing polymer chain"-enzyme complex, and the stability of this complex should be highly correlated to the polymerization degree: when the "growing polymer chain" becomes longer, the stability of the complex decreases. Unfortunately, this hypothesis is not yet sufficiently supported by experimental data. Furthermore, molar mass limitation could also be due to the decrease in polymers solubility as a function of the chain length[149].

All the previously presented data show that there are numerous methods to improve the eROP kinetics, from ultrasonic to microwave activation, through ionic liquids solvent. Individually, each of these methods led to limited improvements, but taken as a whole, they participate in a greater understanding of the reaction, and could hopefully lead to future amazing developments. In addition to the optimization of the reaction itself, there are also many improvements on the process to be made, such as concentration, drying methods, enzymes supporting, reactive extrusion or continuous flow reactions, which represent interesting perspectives to implement these reactions for industrial applications.

Conclusion du premier chapitre

Cet aperçu bibliographique de l'état de l'art en matière de polymérisation enzymatique par ouverture de cycle nous permet de mieux appréhender les paramètres clef de cette réaction et l'influence de nombre d'entre eux sur les caractéristiques des macromolécules finales.

Du point de vue des monomères, cela concerne principalement l'influence de la contrainte de cycle, de la polarité, de la conformation, de l'encombrement ou encore de la stéréochimie des substituants du cycle.

En ce qui concerne les enzymes, cet état de l'art permet d'identifier quelles sont celles qui sont actives et les principales raisons pour lesquelles elles le sont. Il apparait notamment que la structure des sites actifs doit être suffisamment large pour permettre une bonne accessibilité des monomères ainsi que des oligomères et chaînes polymères en croissance. Enfin, un autre paramètre important concerne les phénomènes de positionnement ou d'ouverture/fermeture du couvercle du site actif, qui peuvent, en fonction de l'environnement de l'enzyme, autoriser ou interdire aux substrats l'accès au site actif.

Cet aperçu bibliographique nous a également permis de montrer certaines lacunes dans les connaissances actuelles de la réaction de polymérisation par ouverture de cycle catalysée par les lipases, comme l'influence de la solubilité des monomères, oligomères et polymères dans le milieux réactionnel ou encore l'influence de l'hétéroatome intra-cyclique qui n'a était que très peu décrite.

La suite de ce travail a donc consisté à essayer, à notre échelle, de combler certaines de ces lacunes. Ceci s'est tout d'abord traduit par une étude portant sur la polymérisation de lactone et plus spécifiquement du lactide, permettant de mettre en évidence l'importance des paramètres de solubilité des monomères, oligomères et polymères et celle de l'hydratation du milieu mais permettant également de proposer de nouveaux moyens d'améliorer cette réaction d'eROP. Puis, la suite de nos travaux a concerné l'étude de monomères cycliques portant d'autres hétéroatomes que l'oxygène, que sont les thiolactones pour l'atome de soufre et les lactames pour l'atome d'azote. Enfin, dans la

continuité de l'étude sur les lactames, nos derniers travaux ont porté sur la copolymérisation de lactone avec des acides aminés soufrés.

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III. Chapitre 2 – Cas des lactones, influence de la structure du monomère et assistance par le solvant.

Préambule

Ce chapitre est centré sur l'étude de la polymérisation enzymatique par ouverture de cycle des lactones. Nous nous attacherons ici spécialement à l'étude de l'eROP du lactide en recherchant de nouveaux vecteurs d'amélioration du contrôle des polyacide lactique formés, et de la cinétique correspondante, et en essayant de comprendre les phénomènes mis en jeu pour permettre une rationalisation future de cette réaction de polymérisation.

Ainsi, l'influence de l'ajout de co-solvants actifs, de la quantité d'eau résiduelle présente dans l'enzyme et dans le milieu réactionnel, ou encore du type d'enzyme active en fonction du monomère feront l'objet d'une étude détaillée et approfondie.

Toutefois, avant d'entrer dans le détail de l'étude de ces paramètres, ce chapitre débutera par la présentation d'un certain nombre d'expériences préliminaires. Il s'agit d'une part de prendre en main la méthode de polymérisation enzymatique d'un point de vue pratique et d'autre part de rechercher un système de solvant adapté à la fois à notre principal monomère d'étude, le lactide, et aux lipases étudiées par la suite. Pour cela, nous avons utilisé comme solvant principal le toluène, bien connu pour permettre ce type de réaction enzymatique mais assez médiocre solvant du lactide, auquel nous avons ajouté divers co-solvants afin de faire varier la solubilité du toluène tout en préservant une activité enzymatique notable.

Ensuite, pour clôturer ce chapitre un certain nombre d'expériences complémentaires réalisées parallèlement à l'étude de l'influence de l'ajout de co-solvant, seront présentées. Ces manipulations auront pour but de corroborer certaines des hypothèses présentées dans le premier chapitre.

Il s'agira ainsi de vérifier que plus la contrainte de cycle est faible, plus le cycle est souple et capable de s'adapter à la forme du site actif, et plus la polymérisation enzymatique semble favorisée, aussi au niveau de la vitesse de réaction que de la longueur de chaîne cinétique.

Ces expériences préliminaires permettront également de vérifier le fait que les enzymes ayant un site actif en forme d'entonnoir semblent efficaces pour la synthèse de polylactones[1], car elles permettent l'accès à la chaîne polymère en croissance. En effet, un site actif interne ou en tunnel empêcherait, par des contraintes stériques, aux chaînes en croissance d'être stables dans le site actif de l'enzyme. Ce dernier point et l'étude d'enzymes différant par le type de site actif nous permettra par ailleurs de fixer les enzymes actives qui seront ensuite utilisées dans le reste de l'étude.

Manipulation préliminaire – influence du solvant

Un problème important, lors de la polymérisation enzymatique du lactide, est sa faible solubilité dans les solvants réputés efficients pour cette réaction, comme le toluène ou le diisopropyle éther. En effet, la polarité et la cristallinité du lactide, comme celles du PLA, peuvent les rendre rapidement insolubles aux températures où se font classiquement les réactions de polymérisation enzymatique[2]. Cette solubilisation difficile du monomère associée à des phénomènes de cristallisation *in situ* du polymère formé peuvent rendre la polymérisation enzymatique des lactides assez complexe. Cela peut, en partie, expliquer les résultats mitigés recensés dans la littérature pour ces monomères (à quelques exceptions près).

Ainsi, notre première approche pour améliorer la polymérisation enzymatique de PLA a été d'utiliser différents solvants et mélanges de solvants afin d'améliorer la solubilité des monomères et celle des oligomères et polymères formés, tout en préservant autant que possible la réactivité de l'enzyme.

Table III-1 polymérisation enzymatique du lactide par LBC et N435 dans différents mélanges de *solvants*

enzyme	Système (ratio)	t(h)	C/M (%w)	Substrat	η (%)[a]	Mn [a]
N435	Toluène / DMF (2:1)	48h	10	D-Lactide	31%	300
N435	Toluène / DMSO (2:1)	48h	10	D-Lactide	6%	300
N435	Toluène / THF (2:1)	48h	10	D-Lactide	10%	300
N435	Toluène / AcCN (2:1)	48h	10	D-Lactide	16%	144
LBC	Toluène / DMF (2:1)	48h	5	L-Lactide	-	-
LBC	Toluène / DMSO (2:1)	48h	5	L-Lactide	12%	144
LBC	Toluène / THF (2:1)	48h	5	L-Lactide	7%	300
LBC	Toluène / AcCN (2:1)	48h	5	L-Lactide	-	-

[a] : déterminé par RMN

Les résultats de ces expérimentations, résumées dans le Table 1, ne nous ont pas permis de trouver un meilleur compromis que le toluène pour mener nos réactions de polymérisation. En effet, les mélanges de solvants, bien que permettant une bonne solubilisation du lactide, ne permettent pas d'obtenir une activité enzymatique significative. Aussi, nous utiliserons donc le toluène comme unique solvant pour la suite des expériences.

De ce fait, notre seconde stratégie pour améliorer la réaction de polymérisation enzymatique du lactide a été d'utiliser une base aminée en tant que co-solvant « actif » tout en essayant de contrôler la quantité d'eau présente dans le milieu et en étudiant l'influence d'enzymes différentes présentant un site actif à la structure dite'« entonnoir » comme LBC et CALB ou « tunnel » comme LCR. Les résultats obtenus dans le cadre de cette étude sont présentés ci-après sous la forme d'un article scientifique qui a été publié en 2015 dans la revue *RSC Advances.*

Mixed systems to assist enzymatic ring opening polymerization of lactide stereoisomers

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Introduction

Polylactide (PLA) is one of the most promising biobased and biodegradable polymers[3] , with a large worldwide production, higher than 200 kTons/year. The most wide-spread production process of PLA is a combination of (i) fermentation for producing lactic acid[4,5], and (ii) chemical synthesis[6]. At first, lactide is obtained from lactic acid produced by sugar fermentation followed by the lactide chemical ring opening polymerization (ROP). PLA has been widely studied for 40 years[7] and more extensively during the last decade. PLA is suitable for several applications, from short-term packaging to biomedical purposes.

Nowadays, metallic-based catalysis is the main way to obtain well-controlled PLA[6]. However these catalysts may induce some toxicity (which could be detrimental for e.g., implants or tissue engineering)[8], environmental pollution (in the case of e.g., compostability) and also increase the polymer degradation kinetics[9] (which could limit the material use). Some common organocatalyst for ROP (like DMAP or triflic acid or N-heterocyclic carbene)[10] are efficient but present also some toxicity. Currently, enzymatic catalysis shows a great potential to substitute metal-based catalysts or toxic organocatalysts to limit final toxicity, environmental impacts and preserve abiotic degradation of the polymer, in perfect agreement with a sustainable development and concepts of green chemistry[11].

Lipases (E.C. 3.1.1.3) seem to be the most efficient enzymes for ROP. They can be found in most organisms from microbial, plant and animal kingdom[12]. They are serine hydrolase that catalyze ester bond cleavage in aqueous medium (physiological action is cleavage of triglyceride) and they are also able to catalyze esters bond formation in organic medium (reverse reaction)[13]. By enzymatic catalysis, reactions can be performed under mild conditions (low temperature and pressure, neutral pH) and systems with high catalytic

activity and very good reaction control of enantio-, chemo-, regio-, and stereo-selectivities can be expected. Owing to these advantages, enzymatic processes could provide precise control of polymer architectures, allowing the synthesis of polymers with specific structures using a clean process, without by-products, displaying energy savings[14]. Enzymatic polymerization can thus be regarded as an environment-friendly synthetic process for polymeric materials, providing one of the best examples of "green polymer chemistry".

Enzymatic Ring Opening Polymerization (eROP) of lactones has been studied since 1993[15] but most of the studies concern ε-caprolactone[16] and the few ones on lactide often show inconsistent results[11,17]. It has been reported that lipases with a funnel-binding site, such as lipase B from *Candida antarctica*, are the most appropriate choice for lactone polymerization[1]. However, eROP of lactones still shows some strong limitations: kinetics is usually slow and only some monomers can be polymerized[11].

Some methods to improve the polymerization kinetics have been used, such as continuous flow reaction[18], or ionic liquid as solvent[19], with mixed results. However, the main investigations concerned the immobilization of proteins onto various supports (e.g. acrylic resins, silicones or nanoclays) to increase the enzyme stability and catalytic activity for polyester synthesis[20]. Additional methods to improve lipases activity have also been tested and developed. Esterification, interesterification or transesterification catalyzed by lipases have been studied and some optimizations have been performed on this type of reaction[21]. Some authors studied the influence of the medium composition such as the solvent type[17] or the optimum water content[22,23].

Regarding the lactide enzymatic polymerization, among all existing enzymes, only a few of them have shown an activity, especially lipase B from *Candida antarctica*, lipase from porcine pancreas, lipase from *Pseudomonas fluorescens* and from *Burkholderia cepacia*[24]. Results are usually quite heterogeneous, but most of the time eROP of lactide produces oligomers of low molar mass with poor yield and slow kinetic (reaction time from 1 day to 1 week)[25,26]. To overcome these weaknesses different approaches have been tested such as the copolymerization with better recognized monomers such as ε-caprolactone[27–29] or glycolide[30], the immobilization of enzymes onto nanoclays[27], or special reaction media like

supercritical fluids[31] or ionic liquids[32]. However, most of these methods resulted in rather limited improvements.

Apart from enzymatic polymerization, some authors studied the influence of various organic bases (aromatic tertiary amine with pyridine and some pyridine derivatives, and aliphatic tertiary amine like triethylamine, *N*-methylpiperidine) on lipases activity towards the hydrolytic resolution of esters reporting significant lipase activation with some of the tested bases[33]. Interestingly, to the best of our knowledge, such activation of lipase by organic bases has not been tested and reported so far for lactide eROP.

In the present study, we thus implemented a comprehensive mechanistic approach to improve enzymatic ring opening polymerization of lactide, starting from proposed mechanism and current knowledge to the development of a new approach of base-assisted activation of the monomers and/or the polymer growing chains. The first part was focused on the selection of active enzymes for the eROP of lactide. We selected two lipases with a funnel-like binding site (i) lipase B from *Candida antarctica* immobilized on acrylic resin, which has been extensively studied for this reaction and which is known for being specific of D-lactide[34–36] and (ii) lipase from *Burkholderia cepacia*, which is known to be specific of L-lactide[36]. The influence of the medium water content and drying method on the reaction kinetics and final molar masses was then investigated. Finally, the influence of the addition of an amino base was studied to investigate if such co-solvent could activate the lactide eROP with the aim to further enhance the polymerization kinetics and to reach higher molar masses.

Experimental

Materials: Both D-lactide and L-lactide isomers were obtained from Purac (with the commercial name of Purasorb®) and were purified by sublimation and recrystallization prior use. Novozym®-435 (N435), acrylic resin-immobilized form of *Candida antarctica* lipase B (CALB), was purchased from Aldrich. Lipases from *Burkholderia cepacia* (LBC), from porcine pancreas (PPL) and from *Candida rugosa* (LCR) were purchased as lyophilized powders from Aldrich. Anhydrous toluene was freshly distilled over sodium under nitrogen atmosphere. Anhydrous triethylamine (TEA) was freshly distilled over molecular sieve under vacuum. Other solvents (GC grade) were purchased from Acros and used without further purification.

Enzymatic ring opening polymerization setup:

All reactions were carried out in selected dry solvent or in chosen mixture of dry solvents at 70 and 90 °C, for N435 and LBC, respectively. For that, 6.9 mmol of lactide (1 g) and 4Å molecular sieve (when necessary) were introduced into previously dried Schlenck tube under an inert dry argon atmosphere. The tube was immediately capped with a rubber septum and then immersed in a heated oil bath at 100 °C, until the melting of lactide monomer. Toluene was transferred with a syringe through rubber septum caps. The reaction tube was cooled down to reaction temperature. Predetermined amounts of catalysts, N435 (100 mg) or LBC (50 mg) were quickly introduced in the tube under an inert dry argon atmosphere. The tube was immediately capped with a rubber septum. The enzyme addition marked the beginning (t0) of the polymerization kinetic study. Then, an aliquot was withdrawn at specified time intervals to monitor the polymerization reaction progress. Reactions were terminated by dissolving the reaction mixture in chloroform and when necessary removing the catalyst by filtration. Part of the solvent in the filtrate was then stripped by rotary evaporation at 35 °C. The polymer in the resulting concentrated solution was precipitated in cold methanol (previously stored at −20 °C) to ensure also the recovery of the shortest chains. The PLA precipitate was separated by filtration and dried overnight at 30 °C under vacuum.

System drying procedures:

To minimize the water content in the reaction medium, the monomers were dried by sublimation, the reaction solution medium has been dried using a molecular sieve and the reactor atmosphere was dried by placing molecular sieve or anhydrous calcium chloride in the argon flux tubing. For some experiments, a further drying of the reaction medium was performed by freeze-drying the enzymes for 24 to 36 hours.

Lipases esterification activity measurement:

3.3 mmol of octanol (0.429 g), 3.3 mmol of oleic acid (0.932 g) and 1 mL of toluene were introduced into a reaction tube with a magnetic stirrer under argon atmosphere. A determined amount of enzyme (about 0.1 g) was then added to the reaction medium. The tube was immediately capped with a rubber septum and immersed in a heated oil bath at the selected reaction temperature. After 30 minutes, the reaction was quenched by cooling

in an ice bath and the enzyme was removed by filtration. Reaction extent (ξ) was determined according Equation 1 from 1H NMR spectroscopy by relative integration of characteristic octyl oleate peak (O=C-O-CH_2- t 4.13ppm) and characteristic octanol peak (HO-CH_2- t 3.63ppm), $I_{octyl\ acetate}$ and $I_{octanol}$, respectively

$$\xi = 1 - \left(\frac{I_{\text{octanol}}}{I_{\text{octanol}} + I_{\text{octyl oleate}}}\right) \quad (1)$$

Size Exclusion Chromatography (SEC) analysis:

The number-average molar mass (Mn), the mass-average molar mass (Mw) and the dispersity (Đ) of the resulting samples were determined by SEC, using an Agilent PL 50 instrument. This device was equipped with a guard column 50 mm (5 µm) and a 300 mm column (PL Mixed C, 5 µm). Refractive index (RI) detector was used. Tetrahydrofuran (THF) was used as the eluent at a flow rate of 1 mL min^{-1}. The apparatus was calibrated with linear polystyrene standards from 900 to 1,000,000 g mol^{-1}.

NMR measurement:

^{1}H NMR spectra were recorded on a Bruker Ascend™ 400 spectrometer at 400 MHz, with, at least, 256 scans for kinetic measurements. $CDCl_3$ was used as solvent.

Monomer conversions were determined by NMR analyses on quenched aliquot, using Equation 2. This value was determined from the integrals of the lactide characteristic peak (-O-CH-CH_3 q 4.80-4.90 ppm, $I_{lactide}$), the PLA characteristic peak (-O-CH-CH_3 m 5.00-5.15 ppm, I_{PLA}) and chain-end characteristic peak (HO-CH-CH_3 q 4.20-4.30 ppm, $I_{endchain}$).

$$Monomer\ conversion\ (\%) = 1 - \left(\frac{I_{\text{lactide}}}{I_{\text{lactide}} + I_{\text{PLA}} + I_{\text{endchain}}}\right) \times 100 \quad (2)$$

Thermogravimetric analysis (TGA) coupled Fourier transform infrared spectrometer:

Enzymes water contents were determined by TGA coupled with a FTIR for evolved gas analysis. TGA measurements were conducted under dry helium (at a flow rate of 25 mL min^{-1}) using a Hi-Res TGA Q5000 apparatus from TA Instruments. The samples (5-9 mg placed in a platinum pan) were heated up to 450 °C at 5 °C min^{-1}. FTIR spectra were recorded on a Nicolet 380 (Thermo Electron Corporation) by performing 16 scans with 4 cm^{-1} resolution.

MALDI-ToF Mass spectroscopy Analysis:

Sample preparation: matrix solutions were freshly prepared: Super DHB (9:1 mixture of 2,5-Dihydroxybenzoic acid and 2-hydroxy-5-methoxybenzoic acid, from Sigma Aldrich) was dissolved to saturation in a $H_2O/CH_3CN/HCOOH$ (50/50,1%) solution. Typically, a 1:1 mixture of the sample solution in CH_2Cl_2 was mixed with the matrix solution and 1 µL of the resulting mixture was deposited on the stainless steel plate.

Mass spectra were acquired on a time-of-flight mass spectrometer (MALDI-ToF-ToF Autoflex II ToF-ToF, Bruker Daltonics, Bremen, Germany) equipped with a nitrogen laser (λ = 337 nm).

An external multi-point calibration was carried out before each measurement using the singly charged peaks of a standard peptide mixture (0.4 µM, in water acidified with 1% HCOOH).

Scan accumulation and data processing were performed with FlexAnalysis 3.0 software.

Specific rotation measurement:

The $[\alpha]^{20}_D$ measurement was measured by a polarimeter MPC 200 thermostated at 20°C, in a 100mm long cell (diameter = 0.3mm). Measured solution was prepared at 0.9 to 1mg.mL^{-1} in chloroform.

The optical purity (o.p.) was calculated by the following equation:

$$\text{o.p.} = \frac{[\alpha_D^{20}]}{[\alpha_D^{20}]_0} \times 100 \qquad (3)$$

$[\alpha_D^{20}]_0$ is fixed at +150° according to the literature[37].

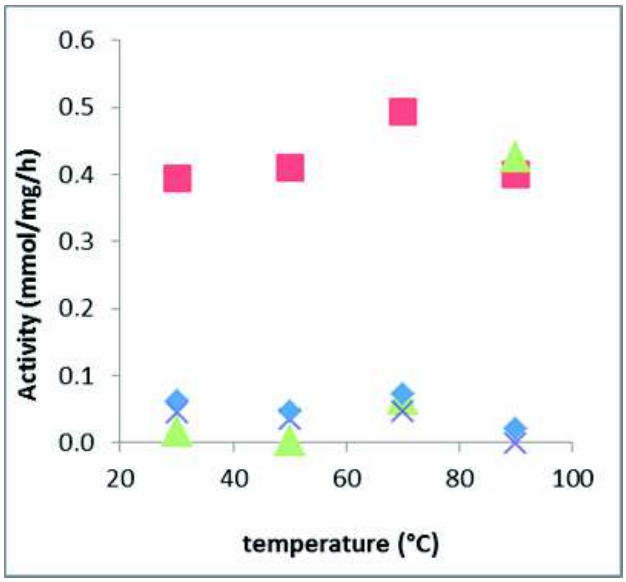

Figure III-1. *Enzymatic activity for olecic acid esterification by octanol (expressed in mmol of ester formed per mg of enzyme and per hour) at various temperatures; ■ N435; ▲ LBC; × LCR; ◆ PPL*

In a preliminary step, the optimal activity temperature was determined for each lipase, by performing a simple esterification reaction (octanol and oleic acid) in toluene, determining the initial reaction rate at different temperatures. Figure 1 shows the activity profiles with optimal temperatures of 90 and 70 °C for lipases from *Burkholderia cepacia* (LBC) and for N435, respectively. These temperatures were thus set as the optimal temperatures for the polymerization reaction.

Two other lipases (PPL and LCR) were also tested since they were previously reported to be efficient for eROP of lactone[38,39]. However in these esterification conditions, PPL and LCR were not active. Because of their poor activity in our conditions, these lipases were not further considered in the next experiments.

Table 2 (experiments 1 to 6) shows that lactides eROP showed mixed results. Only low molar mass oligomers were produced (about 700 to 3500 $g.mol^{-1}$ equivalent PS). These values are significantly lower than those usually obtained by conventional chemical ROP, equivalent for CALB and slightly lower for LBC, than those reported in the literature for eROP[15,24].

Table III-2. Enzymatic ring opening polymerization of lactide isomers catalyzed by lipase from *Burkholderia cepacia* (LBC) and immobilized lipase B from *Candida antarctica* (N435)

entry	enzyme	monomers (1g)	E/M (w)[a]	Solvents (ratio)	T °C	t(h)	Final ξ (%)[c]	η (%)[d]	Mn ($g.mol^{-1}$)[b]	Đ[b]
1	N435	L-Lactide	0,1	Toluene	70	48	23	12	-	-
2	N435	DL-Lactide	0,1	Toluene	70	48	5	-	-	-
3	N435	D-Lactide	0,1	Toluene	70	48	98	90	3500	1,3
4	LBC	D-Lactide	0,05	Toluene	90	48	14	5	-	-
5	LBC	DL-Lactide	0,05	Toluene	90	48	20	10	500	1,1
6	LBC	L-Lactide	0,05	Toluene	90	48	80	65	700	1,1
7	N435	D-Lactide	0,1	Toluene	70	36	98	90	4200	1,2
8	LBC	L-Lactide	0,05	Toluene	90	48	80	65	1000	1,1
9	N435	D-Lactide	0.1	Toluene: TEA (2:1)	70	8	>95%	89%	4300	1.4
10	N435	D-Lactide	0.1	Toluene: TEA (2:1)	70	24	>95%	89%	4900	1.9
11	N435	D-Lactide	0.1	Toluene: TEA (2:1)	70	48	>95%	89%	3800	2.4
12	LBC	L-Lactide	0.05	Toluene: TEA (2:1)	90	8	92%	78%	1500	1.6
13	LBC	L-Lactide	0.05	Toluene: TEA (2:1)	90	24	94%	80%	1800	2.0
14	LBC	L-Lactide	0.05	Toluene: TEA (2:1)	90	48	>95%	85%	1500	2.4

[a] Enzyme/Monomer weight ratio; [b] Determined by SEC, values given in PS equivalent; [c] Final monomer conversion determined by NMR; [d] Recovery yield

The first experiments (table 2, experiments 1 to 6) have shown that each enzyme is specific to one lactide stereoisomer. N435 is specific of D-lactide and LBC is specific of L-lactide. These results are in agreement with current knowledge[34–36]. Although the reaction time has been fixed at a maximum of 48h, if conversion was almost complete and not evolving anymore, the reaction was stopped before. Quantitative reaction was obtained for N435 (conversion greater than 95% after 1.5 days) and quasi-quantitative (about 80% monomer conversion after 2 days) with LBC as catalyst. The recovered PLA yields were of 94 and 81% of converted monomers for N435 and LBC, respectively. The small amounts of converted monomer that are not recovered likely correspond to the shortest chains, which could be eliminated with unreacted monomer during the precipitation step.

Furthermore one can notice that the obtained PLA show very narrow molar mass distribution, with dispersity ranging from 1.1 to 1.3, which means that chain coupling or

other side reactions such as cyclisation, chain scission or chain transfer are negligible (see Figure 2). Significant occurrence of these reactions would induce a larger or even a polymodal molar masses distribution. However, cyclisation or limited chain scission reactions cannot be totally excluded since the corresponding products could be eliminated during the precipitation step as in the case of short oligomers. But this monomodality also indicates that there are no transfer or chain-splitting side reactions or, at least, that such reactions are negligible.

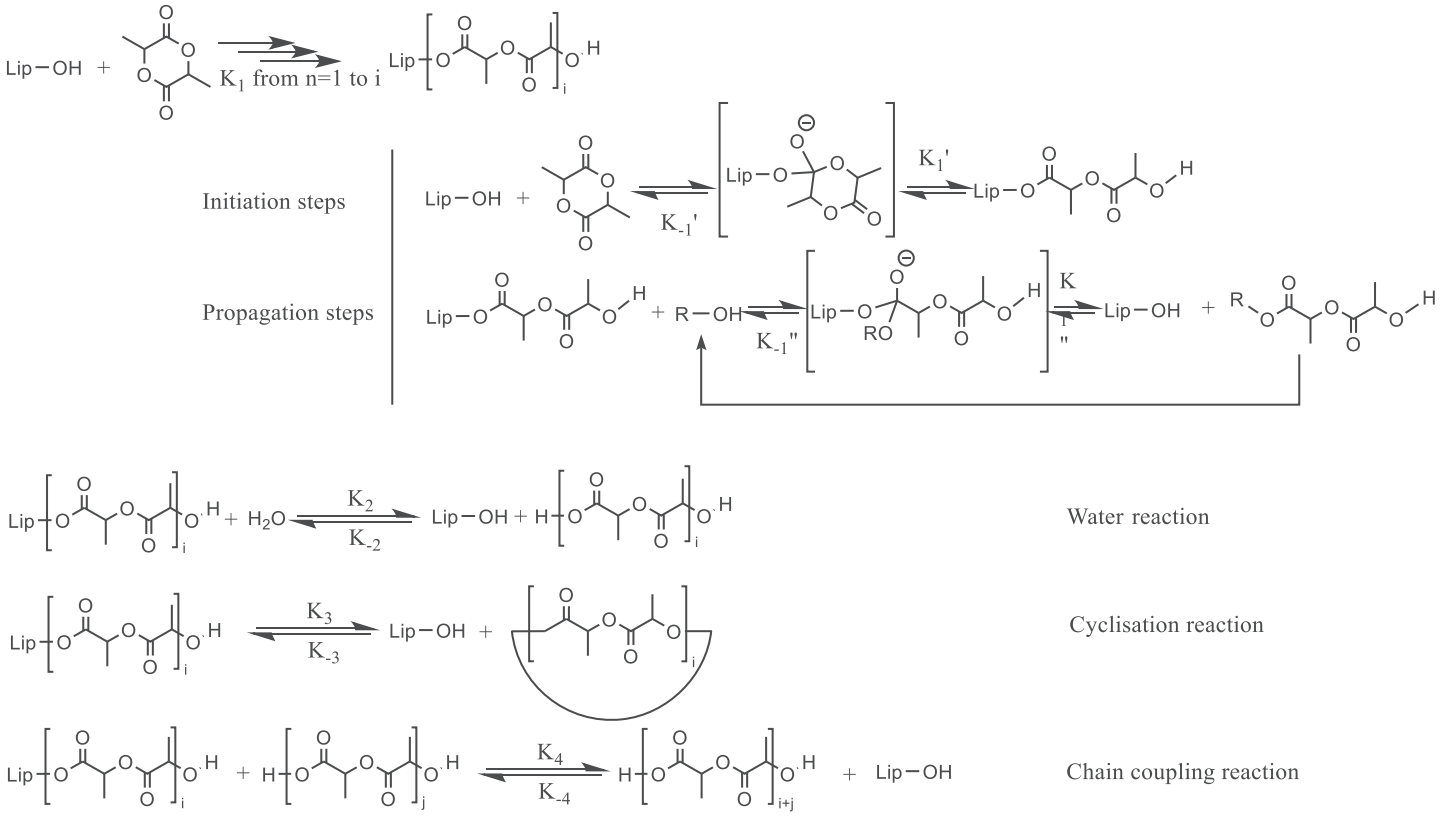

Figure III-2. *Mechanism and reaction pathways for enzymatic ring opening polymerization of lactide, adapted from PM Johnson and coworkers*[40]

These results were partially confirmed by MALDI-ToF mass spectroscopy (see figure 3), that shows a bimodal oligomers distribution respectively centered on 545 g.mol^{-1} (Dp=7) and on 1193 g.mol^{-1}, corresponding to Dp=16 initiated by water molecules (M= 72Dp + $M_{endchain}$ + M_{Na+}, for water initiated reaction end-chain are H and OH). This bimodal distribution cannot correspond to cyclic oligomers and seems to be too narrow for chain splitting reaction but it could mean that two distinct water populations are involved.

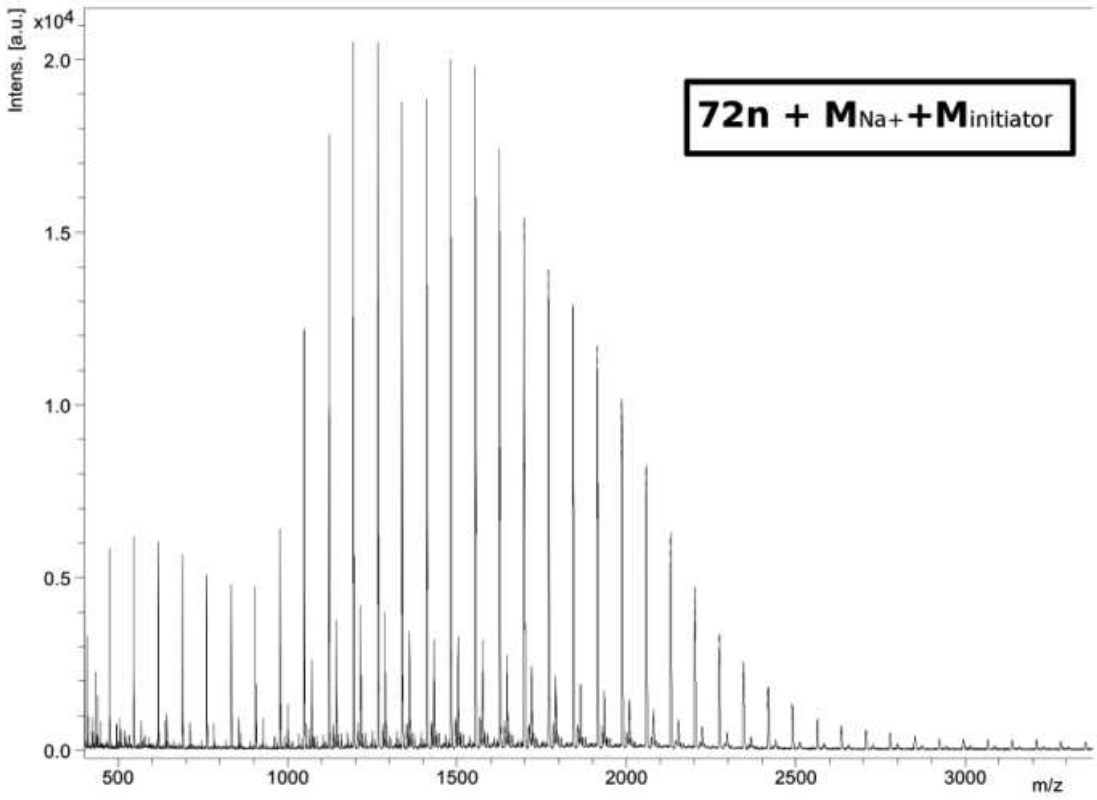

***Figure III-3**. MALDI-ToF MS spectrum of PDLA oligomers synthesized with non-dried N345 as catalyst*

As shown in Figure 4, reactions kinetics are quite slow compared to metal-based catalysts or organocatalysts. To reach 80% monomer conversion, the time required is at least 24 and 48h, for N435 and LBC, respectively. Such slow kinetics are usual for enzymatic reactions on lactide substrate, especially for the enzyme reverse reaction such as lactide eROP[17]. For the eROP of lactide, N435 shows higher results than LBC, with a reaction two times faster, a higher yield (90 instead of 65% for LBC) and a higher PLA average molar mass (five times greater). Such quantitative conversion (80% to total conversion), significant yields, slow kinetics and low molar mass (less than 4000 $g.mol^{-1}$) are in accordance with literature. Some authors explain this low reactivity by a weak affinity between lipases binding site and lactide[40].

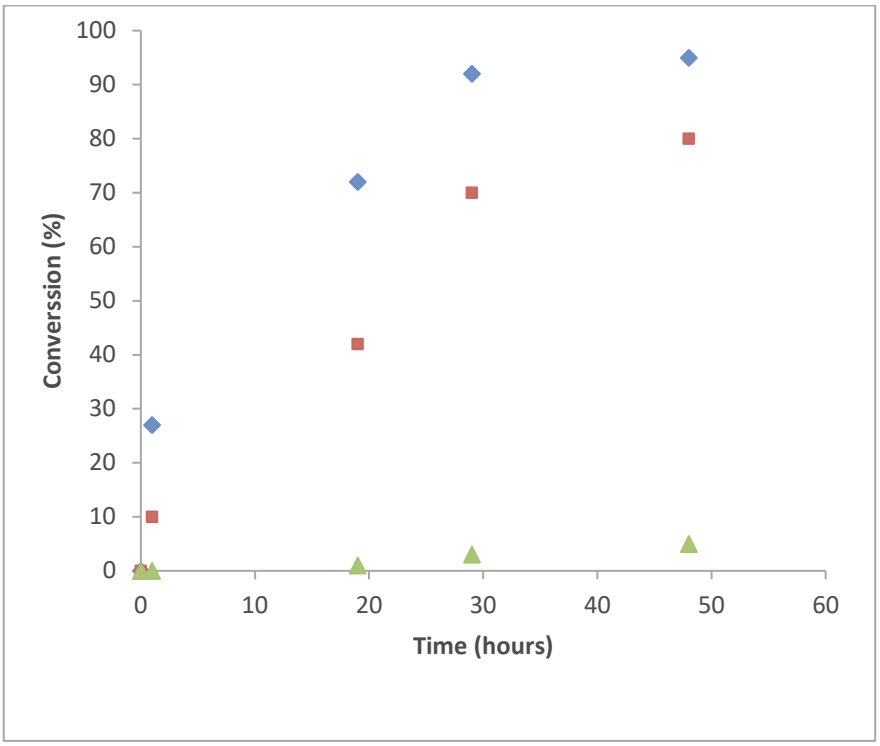

Figure III-4. *Monomer conversion ▲ L-lactide without enzymes, ◆ with N435 –on D-lactide and ■ with LBC on L-lactide*

Influence of the enzymes water content.

As it can be seen from Figure 2, the main parameters to be controlled in order to maximize the PLA chains length are the relative rate of the chain coupling (K2) reaction with regard to the water reaction rate (K4)[39] and the water content in the medium. As described in the experimental section, the monomers, solvent and reactor atmosphere are thoroughly dried. However, the enzyme also brings some water. This last parameter is quite sensitive to control since enzymes drying may cause their denaturation. Enzymes water can by divided in two types: (i) the hydration water and (ii) the structural water[40,41]. Thus, the freeze-drying method was tested to remove hydration water while keeping the structural water in order to preserve enzymes efficiency. The enzymes final water contents were determined by thermogravimetric analysis coupled with infrared spectroscopy and the results are shown on Figure 5.

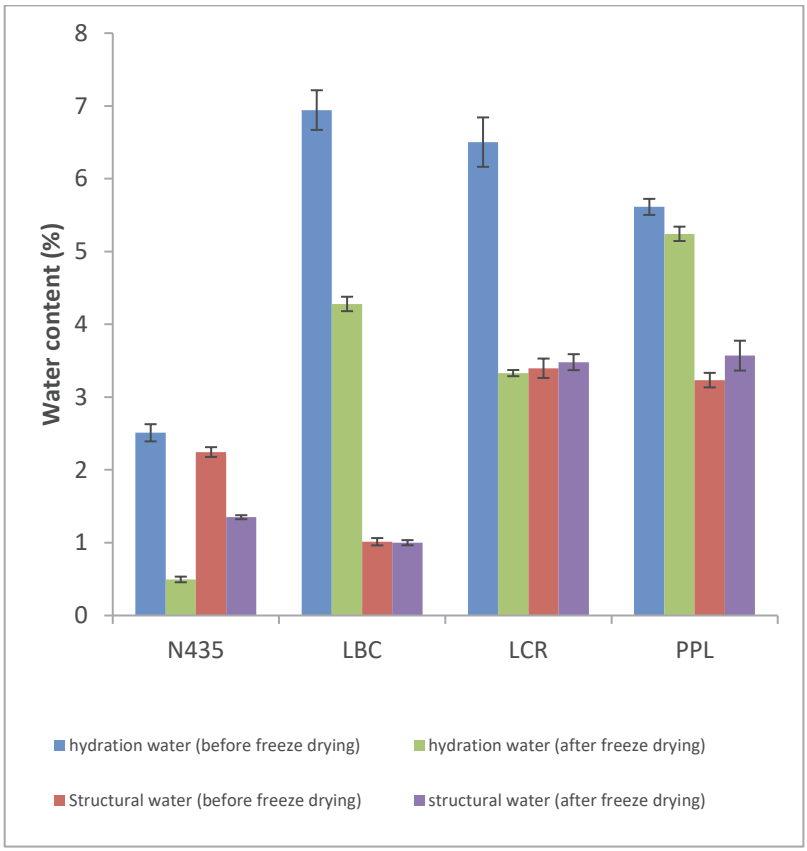

Figure III-5. *Hydration and structural water contents of the lipases, before and after freeze-drying.*

The freeze-drying method allowed a significant reduction of hydration water level, without having a negative impact on the structural water content. This has been confirmed by testing the efficiency of the freeze-dried enzymes for lactide eROP.

As expected this reduction in the hydration water content of the enzyme allowed increasing significantly the final PLA average chain length from 3500 to 4200 $g.mol^{-1}$ for N435 and from 700 to 1000 $g.mol^{-1}$ for LBC, with a corresponding short increase in the reaction time. 80% of conversion was reached after 28h (instead of 24h) and 50h (instead of 48h) for N435 and LBC, respectively. These reactions gave very similar final conversion (>98% for N435 and 80% for LBC) and yield (about 90% and 65% for N435 and LBC, respectively) than the previous experiments without drying. As previously explained, the difference between conversion and yield could be explained by the loss of the short oligomers during the precipitation step. One can also notice that, for N435 the control of these key parameters to minimize the water content allows to obtain PLA chains with narrower molar mass distribution (Đ =1.3 in the first experiments compared to 1.1 in this case). As far as freeze-dried LBC is concerned, Đ

does not significantly change (1.1 to 1.2) but remains very low. As previously mentioned, such low Đ likely attests for the absence of side reactions such as chain transfer, chain coupling or cyclisation.

The MALDI-ToF results (see figure 6) show a monomodal distribution centered on 1481 $g.mol^{-1}$ (Dp=20, water initiated) and show the disappearance of the first oligomer population (see figure 3) which confirms that such population is likely due to the hydration water of our enzymes.

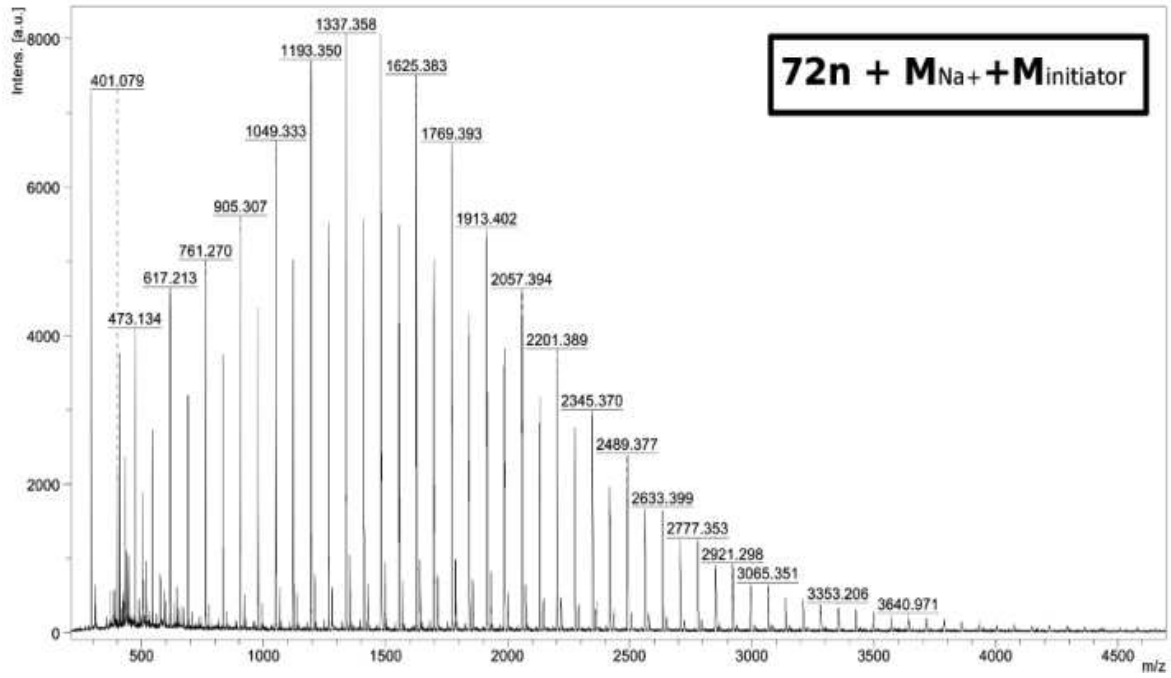

Figure III-6. *MALDI-ToF spectrum of PDLA oligomer synthesized with dry N435*

In all the cases, the molar mass values reach a kind of plateau at 4500 $g.mol^{-1}$. This could be due to the important difference in the chemical structure between natural substrate of the lipase (i.e., fatty acids and triglycerides) and the lactide monomers and oligomers, which are also more polar and more rigid molecules. This may result in a strain for the PLA growing chains to enter and being sufficiently stable in the lipase active/binding site and thus to incorporate additional monomer units.

Study of the activation of lactide eROP with TEA.

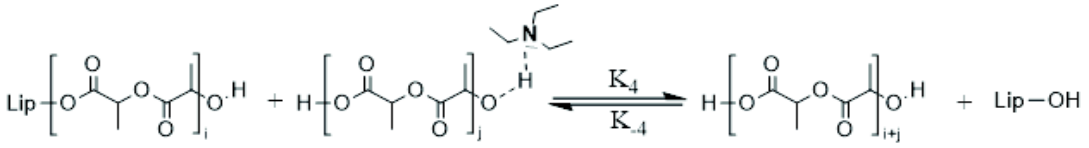

Figure III-7. *Oligomers chain-end activation*

Our second approach to increase the PLA molar mass was based on the increase of the relative rate constant of coupling chain reaction (K4/K2). For that, an amino base, TEA was added as a co-solvent in the reaction mixture with the aim to activate chain-ends of preformed oligomers. Since TEA is slightly basic and nucleophilic, it could activate the monomer for its ring opening and thus increase the reaction rate[25] (Figure 7). However, this co-solvent can also activate chain-splitting reactions.

Figure 8 shows the main results of the addition of TEA with a great impact on the reaction kinetics. Reaction time was reduced about 5 to 6 times for both selected lipases (80% conversion reached after 4 hours for N435, and after 2.5 hours for lipase from *Burkholderia cepacia*). As expected, TEA alone shows some catalytic activity but does not lead to a significant extent of lactide ROP.

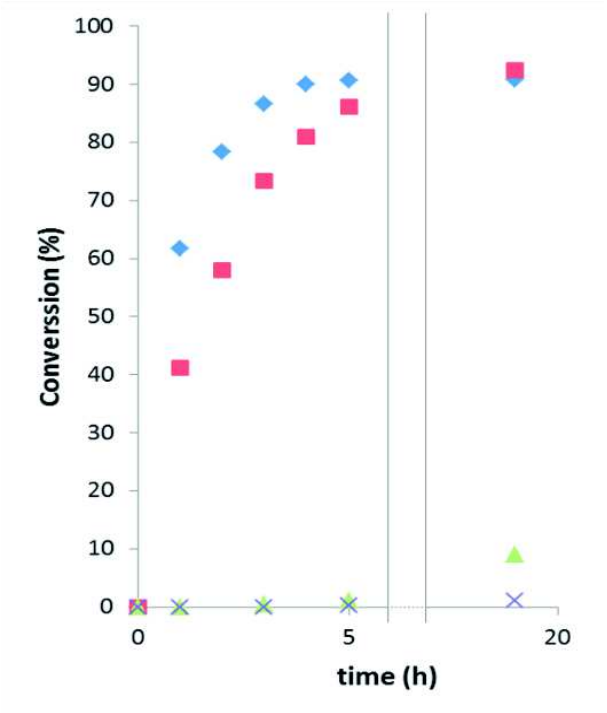

Figure III-8. *Influence of TEA addition on kinetics of eROP catalyzed by LBC (with L-lactide) at 90 °C (◆), N435 (D-lactide) at 70 °C(■), without catalyst (L-lactide) at 90 °C(▲), with neither catalyst nor TEA (L-lactide) 90 °C (x)*

This increase in the reaction rate is probably not only the result of the relative increase in the chain coupling reaction rate but some other side reactions may also have occurred. Tertiary amines like TEA can also promote the epimerization of lactide especially when present in such large amount like in our case. This could affect the polymerization since it is well known that meso lactide is more favorable for enzymatic polymerization by CALB[41].

Table III-3. *Specific rotation of some synthesized PLA n function of reaction time*

Entry[a]	Solvents (ratio)	t(h)	$[\alpha]^{20}_D$	o.p (%)[b]		
7	Toluene	36	114.4	76%		
9	Toluene: TEA (2:1)	8	88.5	59%		
10	Toluene: TEA (2:1)			24	49.8	33%
11	Toluene: TEA (2:1)			48	24.5	6%

[a] see table 1 for details of each entry; [b] optical purity;

To verify the occurrence and the importance of epimerization polarimetric measurements were performed on some of the PLA synthesized (see Table 3). It has been found in the

literature that specific rotation of enantiopure PLA is about $[\alpha]^{20}_D$ = +/-150° (+ for the D form and – for L form)[40]. One can note that the eROP of lactide in toluene already caused some epimerization (o.p. 75%). The addition of TEA induces an important reduction in o.p. (decreased to 6% after 48h hours) but this decrease is too slow (only 17% reduction after 8hours) to explain the increase in reactivity. Furthermore the important reduction in the o.p. after 48h matches with an increase in the dispersity and a lowering of the Mn value. These results confirmed the epimerization of lactide, but the decrease of $[\alpha]^{20}_D$ after the total consummation of lactide indicates that TEA also promotes the racemization of the formed oligomers.

The SEC results (Table 2, experiment 10 and 13) show a significant improvement in the average molar masses, with the addition of TEA, with a 17% increase for N435 catalyst and over 80% increase for LBC. However, one can notice an important increase in dispersity (Đ =1.9 and 2.1, instead of 1.15 and 1.1 in previous experiments, respectively), which may indicate the occurrence of, at least, another type of reaction. The SEC chromatograms show a bimodal profile (see Figure 9) with a marked shoulder corresponding to an average molar mass, twice the one of the main peak.

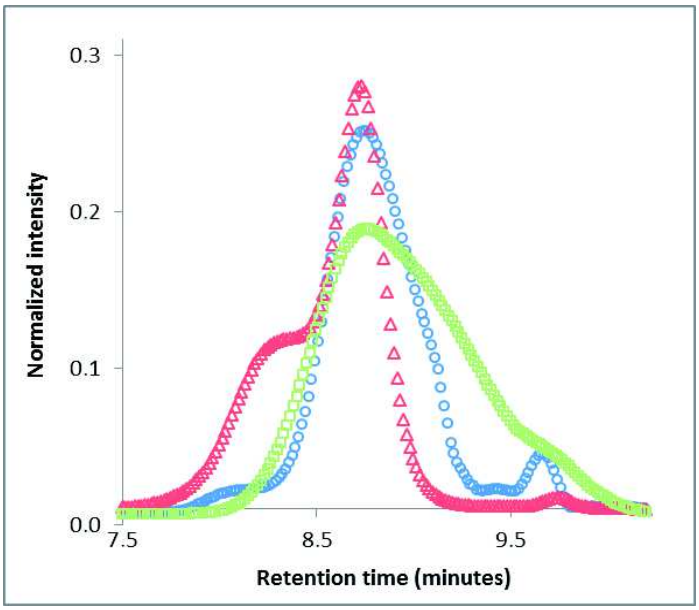

Figure III-9. *SEC elution profiles of PLA produced by eROP catalyzed by N435 in toluene:triethylamine solvent mixture. After 8h (○), 24h (△) and 48h (□) of reaction. All curves were normalized on flow marker intensity.*

Such a doubled molar mass value seems to confirm that chain coupling reaction has been promoted by TEA addition as a result of the chain-end activation of the oligomers.

However, chain scission and chain transfer reactions have also been confirmed by additional experiments. As shown in Table 2 (experiment 9 to 14), average molar masses increase until 24 hours of reaction until a probable progressive inactivation of our catalytic system. Then, for long reaction times, chain scission reactions and chain transfer are likely responsible of the gradual reduction of the average molar mass. Such decrease in Mn values with transesterification reactions for long reaction times is in perfect agreement with previously reported results[42]. One can also notice a small peak or shoulder at about 9.7 minutes retention times, which could be due to cyclic oligomers, as it has already been reported in literature[43].

The activation of coupling chain reaction could also explain the obtained MALDI-ToF spectrum (see figure 10) which shows a superposition of 2 distribution one centred on 1481 g.mol^{-1} (Dp=20, water initiated) and the other centred on 3496 g.mol^{-1} (Dp=48, water initiated) which is 2.4 times the first one.

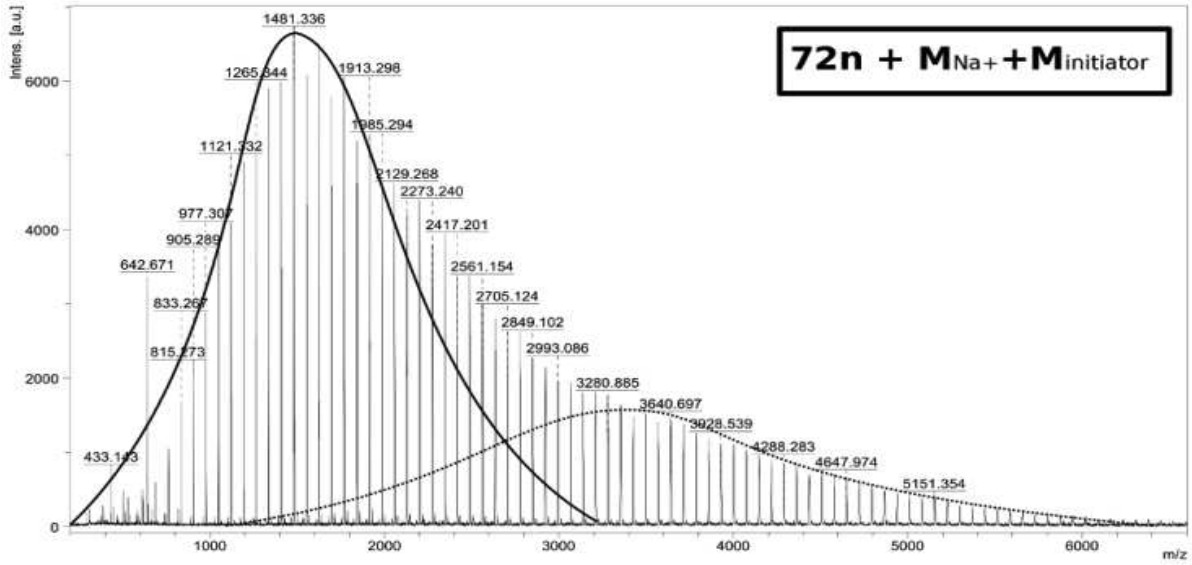

Figure III-10. *MALDI-ToF spectrum of PDLA oligomer synthesized with dry N435 in toluene:TEA mix*

The chain-end activation of the oligomers cannot totally explain our results, especially the kinetic improvement of eROP, and is probably not the only activation way. The influence of

TEA is more likely much more complex. One can consider several additional activation mechanisms. Three simple possible activation mechanisms can be rapidly identified and are described hereafter.

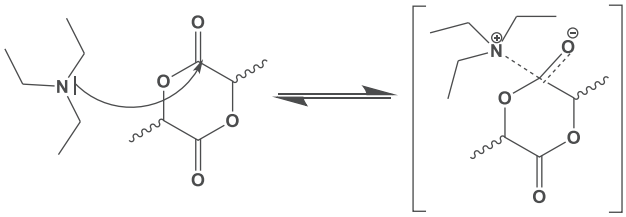

Figure III-11. *Nucleophilic activation of lactide*

Similarly to the nucleophilic activation of the oligomers chain-end, one can consider that TEA could activate the lactide monomer through a nucleophilic attack on its carbonyl carbon (see Figure 11). This would induce an elongation of the O-C=O bond facilitating the ring opening of lactide and then the formation of an O-acyl reactive intermediate.

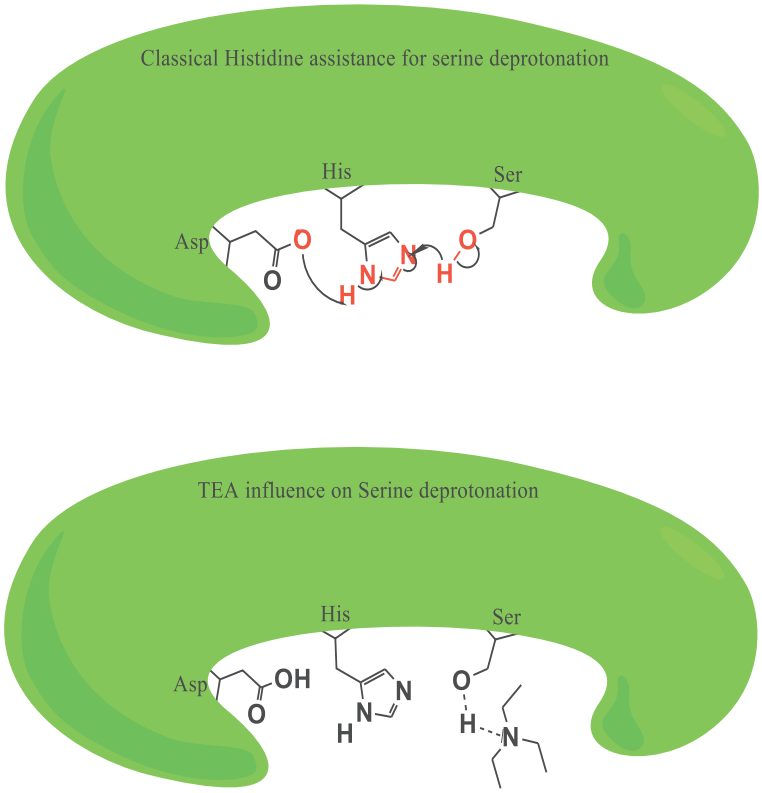

Figure III-12. *Lipase activation by facilitating serine deprotonation.*

A second possible activation way could consist in the basic activation of the serine amino acid of the binding site. As proposed and shown in Figure 12, this would facilitate the serine (Ser) deprotonation and thus increase the reactivity of lipases. In this case TEA would play a role very similar to that of histidine (His) in the enzyme binding site.

The third possible activation mechanism could also come from the nucleophilic character of TEA which could have an impact on the chain backbone through nucleophilic attack on the carbonyl of the ester bonds (Figure 13).

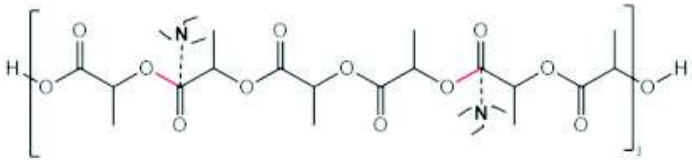

Figure III-13. *Nucleophile activation of oligomers esters bond.*

This last effect could result in increased occurrence of chain-transfer and chain scission reactions that could explain the molar mass decrease we observed (see Figure 9). Besides, we confirmed the chain-splitting by a simple experiment: some previously formed oligomers (3500 $g.mol^{-1}$, Đ=1.3) were added to a mix of toluene and TEA and after 48h at 70 °C, we observed a significant lowering of initial molar mass and a significant increase in dispersity (2100 $g.mol^{-1}$, Đ=2.5).

Whatever the involved activation mechanisms and whatever the enzyme we used, TEA has a clear and positive impact on eROP of lactide on both aspects, kinetic and final molar mass, with a controlled dispersity.

Conclusions

In conclusion, this work reports enantioselective eROP of the two main enantiomers of lactide. Enantioselectivity of selected enzymes have been confirmed, with a selectivity towards D-lactide for N435 and towards L-lactide for LBC. The ability of freeze-drying to reduce enzymes hydration water while preserving their structural water has been demonstrated and polymerization tests confirmed that reducing the hydration water content results in increased oligo-PLA molar masses (Mn increase of 20 and 40%, for N435 and LBC, respectively).

It has also been demonstrated that eROP of lactide is activated by the addition of TEA as an aprotic amino base and that this kind of activator is efficient for both lipases we used. TEA addition permits a significant kinetic improvement (reaction was five time faster for N435) and also increased the molar mass (17% for N435). The kinetic improvement of eROP allows new opportunities for lipases reverse catalysis. In addition with others improvement methods, like continuous flow reaction and/or sonic activation [16-19], this biocatalyst could become competitive with classical organometallic catalysts for some application.

Four possible mechanisms have been proposed for the eROP activation by TEA: (i) a nucleophile activation of lactide, (ii) basic activation of lipases, (iii) basic activation of oligomers chain-end, and (iv) nucleophile activation of esters bond in the oligomers backbone. Our results seem to attest for the occurrence of at least two of these activation ways: the chain-end activation and the activation of ester bond in the chains backbone. However, one can say that a combination of these four mechanisms more likely occurs.

Our future works, by performing complementary experiments and numerical simulation, should allow us to describe and determine the contribution of each activation mechanism on the TEA activated eROP of lactide.

Pour clôturer ce chapitre et compléter ces travaux sur l'étude de l'eROP des lactones, nous présentons ici un certain nombre d'expériences complémentaires. Celles-ci permettront de vérifier certaines des hypothèses proposées dans le premier chapitre. Elles porteront donc sur l'étude de la contrainte de cycle et dans une moindre mesure sur l'influence de la structure du site actif des lipases.

Etude complémentaire : influence de la contrainte de cycle

Pour ces expériences sur l'effet de la contrainte de cycle nous avons choisi trois lactones permettant de représenter de manière globale les principales contraintes de cycle rencontrées dans ce type de molécule (Figure 14). Ces trois lactones sont : la γ-butyrolactone, un cycle à 5 chaînons très rigide, la ω-pentadécanolactone (ou pentadécanolide) un cycle à 16 chaînons où la contrainte de cycle est pratiquement nulle malgré la présence de l'ester intra-cyclique et enfin la ε-caprolactone, qui présente une contrainte de cycle relativement faible (comparativement au cycle plus petit) et donc une conformation plus libre sans toutefois que celle-ci soit totale comme c'est le cas pour le pentadecanolide. Nous profiterons également de ces expérimentation pour comparer l'activité d'une enzyme au site actif en « tunnel », LCR, avec une enzyme au site actif en « entonnoir », CALB.

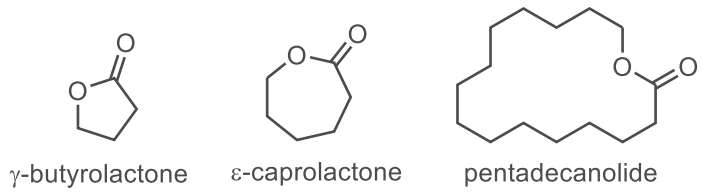

Figure III-14. *lactones utilisées pour étudier l'influence de la contrainte de cycle*

Les résultats obtenus lors de ces expériences sont résumés dans la Table 4. Ceux-ci sont encore bien plus marqués que ceux rapportés dans la littérature. En effet, nous n'avons observé aucune conversion dans le cas de la γ-butyrolactone alors que, comme attendu,

nous obtenons facilement un polymère dans le cas de l'ε-caprolactone et de la ω-pentadécanolactone.

Table III-4. *eROP de lactones de différentes tailles de cycle par une enzyme au site actif en « entonnoir » : CALB et une enzyme au site actif dit « tunnel » : LCR*

Monomère	enzyme	temps	température	solvant	η (%)	Mn ($g.mol^{-1}$)[c]	Đ[c]
γ-butyrolactone	CALB[d]	48h	70°C	toluène	<0,1%[b]	n.m	n.m
γ-butyrolactone	LCR[e]	48h	70°C	toluène	<0,1%[b]	n.m	n.m
ε-caprolactone	CALB[d]	12h	70°C	toluène	93,5%[a]	21 000	1,4
ε-caprolactone	LCR[e]	48h	70°C	toluène	0,5%[b]	n,m	n.m
ω-pentadécanolactone	CALB[d]	12h	70°C	toluène	95%[a]	30 000	1,5
ω-pentadécanolactone	LCR[e]	48h	70°C	toluène	0,5%[b]	n.m	n.m

a : rendement final après précipitation, mesuré par gravimétrie ; b : mesuré par RMN avant précipitation ; c : mesuré par SEC ; d : Lipase B de Candida antarctica ; e : Lipase de Candida rugosa

Toutes ces expériences ont été faites dans les conditions décrites en partie expérimental de ce chapitre.

Outre le fait que la rigidité du cycle semble être, comme attendu, un élément déterminant de la réactivité, on constate l'impossibilité, dans ces conditions, d'obtenir des polylactones avec l'enzyme au site actif en forme de tunnel qu'est la lipase de *Candida rugosa.* Ce résultat est un peu surprenant puisque cette enzyme est connue pour catalyser efficacement la synthèse d'esters d'acide gras et qu'elle a par ailleurs déjà été décrite comme active en ROP des lactones[44]. Toutefois, pour cette lipase les temps de réaction sont généralement bien plus longs que ceux utilisés ici et les rendements bien que supérieurs à ceux que nous obtenons ici ne sont généralement pas très élevés (21% après 240h de réaction)[44].

Conclusion du second chapitre

Dans ce second chapitre, axé sur la polymérisation enzymatique des lactones et principalement du lactide, nous nous sommes attachés à l'étude de plusieurs paramètres. Ainsi, le type d'enzyme et ses conditions optimales pour l'eROP ont été étudiés notamment en termes de solvant, de température et d'hydratation de l'enzyme dans un premier temps puis en termes de sélectivité dans un second temps via une étude sur les énantiomères du lactide.

Ainsi, le solvant principal idéal pour l'eROP du lactide s'est révélé être le toluène qui, bien qu'étant un solvant médiocre pour le lactide comme pour le PLA, est le seul qui a permis la réaction d'eROP. Nous avons pu déterminer que parmi notre panel d'enzymes seules deux d'entre elles étaient significativement actives en eROP du lactide : la lipase de *Burkholderia cepacia* et la lipase B de *Candida antarctica*. Pour chacune de ces enzymes, les températures optimales pour une réaction d'estérification ont été déterminées : 90°C pour LBC et 70°C pour CALB. Par ailleurs, le contrôle de l'eau d'hydratation de ces enzymes par lyophilisation a permis une amélioration significative de la masse molaire des oligomères (augmentation du Mn de 25 à 40% pour CALB et LBC respectivement).

Nous avons également travaillé sur l'activation de la réaction en elle-même et nos travaux ont permis de mettre en évidence une méthode d'activation de la réaction d'eROP du lactide par ajout d'une base aminée, en tant que co-solvant actif. Ainsi, l'ajout de triéthylamine (TEA) comme base a montré des résultats intéressants sur la cinétique de réaction en divisant par cinq le temps de réaction nécessaire pour atteindre 80% de conversion. De plus, ce co-solvant actif semble avoir également un effet sur la structure des macromolécules formées, faisant apparaitre une seconde population de chaînes polymères de plus hautes masses molaires. Toutefois, en contrepartie de ces améliorations, l'ajout de cet activateur a également provoqué une racémisation partielle du lactide et du PLA. Cela nous a permis de formuler des hypothèses relatives au mécanisme d'activation impliqué : (i) activation nucléophile du lactide, et (ii) activation basique de la serine et des alcools terminaux des chaînes en croissance.

Enfin nous avons pu confirmer en partie l'hypothèse formulée dans le premier chapitre, au vu des résultats présentés dans la littérature, sur l'influence de la contrainte de cycle en montrant que cette dernière constitue bien le paramètre prédominant de la réactivité des monomères en polymérisation enzymatique.

Ce second chapitre a permis de mieux appréhender la polymérisation des lactones par voie enzymatique et ainsi de réduire notre champ d'investigation tout d'abord par une sélection des enzymes actives et ensuite par une sélection de solvant. Ce chapitre nous a également permis de mieux comprendre cette réaction, et d'orienter la suite de nos travaux vers des chemins peu explorés jusqu'alors avec une approche novatrice.

Cette étude de l'eROP des lactones a permis (i) des améliorations des systèmes étudiés par rapport à l'existant et (ii) une compréhension plus fine de l'ensemble des mécanismes mis en jeu. Ceci pourra être développé par la suite par d'autres approches, telle que la modélisation moléculaire. Pour notre part, nous avons choisi de poursuivre cette étude de l'eROP en faisant varier l'hétéroatome intra-cyclique afin d'étudier son influence sur la réaction de polymérisation enzymatique mais également d'accroitre les possibilités d'applications de ce type de catalyse dans le domaine des polymères.

Ainsi, le chapitre 3 portera sur la polymérisation de thiolactone par ouverture de cycle enzymatique qui sera adaptée à la copolymérisation avec des lactones.

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IV. Chapitre 3 – Etude de la variation de l'hétéroatome intra-cyclique : le cas des thiolactones

Préambule

Cette partie sera axée sur la polymérisation d'un monomère thiolactone, afin d'évaluer l'influence du changement d'hétéroatome sur la réaction tant du point de vue cinétique que du point de vue de l'architecture des copolymères obtenus.

Il avait tout d'abord été envisagé d'utiliser les équivalents soufrés du lactide et du glycolide, toutefois ces produits sont, a priori, peu stables et les quelques synthèses décrites dans la littérature présentent de faibles rendements[1]. Nous avons donc pris le parti d'étudier l'influence de la substitution de l'oxygène intra-cyclique par un atome de soufre avec le monomère le mieux connu en polymérisation par ouverture de cycle enzymatique, à savoir l'ε-caprolactone et son analogue soufré, l'ε-thiocaprolactone (thiepan-2-one).

Ce monomère n'étant pas commercial, nous l'avons synthétisé. Deux stratégies ont été utilisée pour cela, l'une issue de la chimie traditionnelle (voie A) et l'autre faisant intervenir la synthèse enzymatique de ce monomère (voie B), présentées en Figure 1.

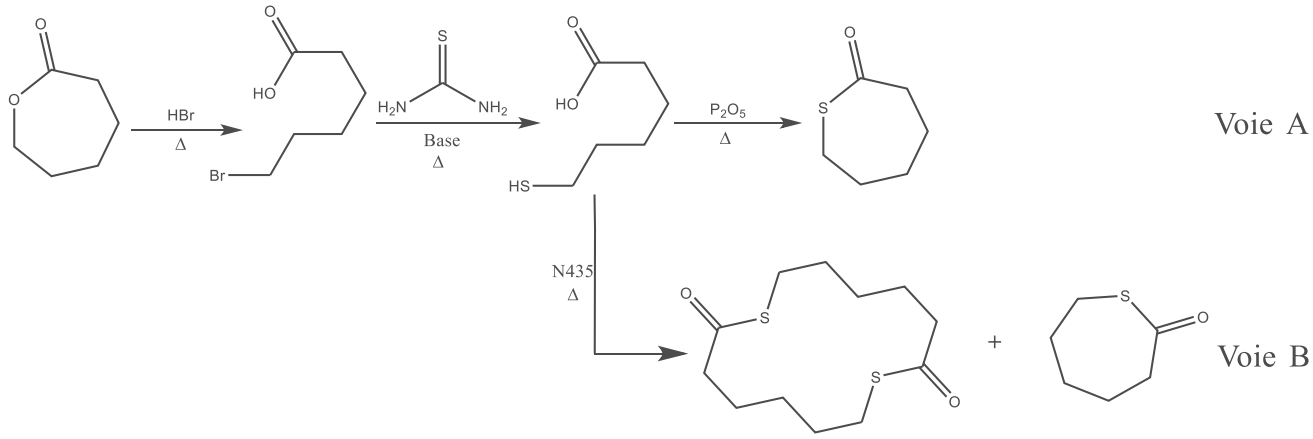

Figure IV-1. stratégies de synthèse de la ε-thiocaprolactone ; voie A[2] et voie B[3]

La synthèse enzymatique (voie B) des thiolactones a été développée dans un premier temps. Cette voie est intéressante et prometteuse pour un mettre en place une approche de chimie entièrement verte « du berceau à la tombe » pour la synthèse des polymères. De plus, cette voie permet d'envisager une synthèse continue du thioacide au polymère en « one pot ». Toutefois, les résultats obtenus ont été très mitigés et donc dans la suite de l'étude, seule la voie chimique traditionnelle (voie A) a été développée. En effet, celle-ci présente de nombreux avantages pratiques, telles qu'une réaction rapide, environ 60 minutes contre 48h pour la voie B, pour un rendement équivalent d'environ 50% après purification et une purification aisée (simple lavage à l'éther et distillation, alors que la voie B nécessite une purification par chromatographie).De plus, cette voie présente également l'avantage de ne pas produire de di- ou de tri-ε-thiocaprolactone (macrocycles), contrairement à la voie B. Cela évite une séparation qui pourrait s'avérer complexe tant ces composés sont chimiquement proches.

Ce monomère a, par la suite, été polymérisé et copolymérisé avec l'ε-caprolactone suivant deux stratégies. La première stratégie consistait en la copolymérisation directe du mélange des deux monomères alors que la seconde, indirecte, avait pour objectif d'obtenir des copolymères avec une microstructure mieux contrôlée.

La suite de ce chapitre décrit donc, sous la forme d'un scientifique qui a été publié en 2017 dans le « *European Polymer Journal*», nos travaux sur la polymérisation de la ε-

thiocaprolactone et sa copolymérisation avec l'ε-caprolactone dans le but de mieux comprendre l'influence que peut avoir l'hétéroatome sur la réaction de polymérisation enzymatique par ouverture de cycle, à la fois d'un point de vue mécanistique que du point de vue de la structure des macromolécule finales obtenues.

Enzymatic synthesis of poly(ε-caprolactone -co- ε-thiocaprolactone)

Article publié sous la référence suivante : S. Duchiron, E. Pollet, S. Givry, L. Avérous, "Enzymatic synthesis of poly(ε-caprolactone -co- ε-thiocaprolactone)", *European Polymer Journal. Vol. 87 pp. 147 - 158,* 2017

Introduction

Poly(ε-caprolactone) (PCL) is a very well-known biodegradable and biocompatible polymer[4,5], suitable for environmental and biomedical purposes. However this biodegradable polyester shows some strong drawbacks such as poor thermal properties and poor chemical solvent resistance[6]. Since several decades, the most conventional PCL synthesis way is the lactone ring opening polymerization (ROP) catalyzed by organometallic compounds, like aluminum, zinc or tin derivatives[7,8]. However these catalysts may induce some toxicity (which could be detrimental for biomedical applications such as implants or tissue engineering)[9], environmental pollution (e.g., in the case of compostability) and also increase the polymer degradation kinetics[10] which could limit the material lifetime. PCL can also be produced by organocatalyzed polymerization with 4-dimethylaminopyridine (DMAP) or 1,5,7-triazabicyclodec-5-ene (TBD) derivatives[11] but those catalytic systems may present also some toxicity. Enzymatic Ring Opening Polymerization (eROP) is another useful method for PCL synthesis[12]. Currently, enzymatic catalysis shows a great potential to substitute metal-based catalysts or toxic organo-catalysts and to limit final toxicity, environmental impacts and abiotic degradation of the polymer, in perfect agreement with a sustainable development and concepts of green chemistry[12]. With enzymatic catalysis, reactions can be performed under mild conditions (low temperature and pressure, neutral pH). Besides, very good reaction control of enantio-, chemo-, regio-, and stereo-selectivities can be expected. Owing to these advantages, enzymatic processes could provide precise control of polymer architectures, allowing the synthesis of polymers with potentially specific structures using a clean process, without by-products, displaying energy savings[13]. Enzymatic polymerization can thus be regarded as an environmental-friendly synthetic process for polymeric materials, providing one of the best examples of "green polymer chemistry".

Lipases (E.C. 3.1.1.3) seem to be the most efficient enzymes for ROP. They can be found in most organisms from microbial, plant and animal kingdom[14]. They are serine hydrolases that catalyze ester bond cleavage in aqueous medium (physiological action is cleavage of triglyceride) and are also able to catalyze ester bonds formation in organic medium (reverse reaction)[15]. The catalytic efficiency depends on the lipases 3D structures. The literature has for instance, reported that lipases with a funnel-binding site, such as lipase B from *Candida antarctica*, are the most appropriate choice for lactone polymerization[16]. However, eROP of lactones still shows some strong limitations: kinetics is usually slow and only some monomers can be polymerized[17].

Lactones eROP has been studied since 1993[18] . Most of the studies concern the opening of the ε-caprolactone[19] and there is only few experiments with other heteroatom like thiolactone[20] or lactam[21]. The introduction of another heteroatom usually modify the final material properties. For instance, polymers with sulfur atom in their backbone show good thermal properties (higher melting and degradation temperatures) and better chemical solvent resistance compared to the equivalent polyesters[22]. By comparison, poly(ε-thiocaprolactone) (PTCL) shows Tm around 105 °C[2] while PCL has a Tm around 60 °C. Interestingly, thioester bonds are relatively common in natural products (natural functionalization of cysteine in protein and biological transition product) and there are some ways to biosynthesize polythioesters[23,24]. Then, polythioesters, especially poly(ε-thiocaprolactone), are potentially biocompatible and could be used to improve or tune thermal and mechanical properties of their polyesters counterparts for biomedical applications.

Only some studies have been published on the synthesis of polythiolactone. But, most of them are based on conventional chemical catalyst[2,25,26]. However, thioester function is chemically quite close to the ester function and enzymatic reaction could show a great potential. Enzymatic trans-thio-esterification have been used in organic synthesis reactions[27-31], for waxes synthesis for example[32]. However, eROP of cyclic thioesters has been rarely studied so far, to produce a large range of polymers architectures. Matsumura et al. proved that this kind of function can be polymerized by lipase with promising results[3,20]. Other authors performed eROP of thiolactones, such as Wu et al with beta-thiolactone[31] or Weber et al. with macrothiolactone[33]. These works are promising but till now there are still

several unanswered questions, many unknown parameters thus requiring further investigations.

The aim of this paper is to study the enzymatic ring opening copolymerization of ε-caprolactone and ε-thiocaprolactone by a one-step and a two steps strategy. Both strategies were applied with the aim to obtain different macromolecular architectures, a more random copolymer with median properties is expected for the one-step strategy while a more blocky structure is pursued for the two-steps strategy. The chemical structures of the corresponding copolymers have been investigated by several methods as 2D NMR and MALDI-ToF MS spectroscopy. The main target of this study based on different synthesis pathways is to control, in a certain extent, the macromolecular architectures of the copolymers synthesized by eROP and to obtain new information on the corresponding reactional mechanisms.

Experimental section

Materials:

ε-caprolactone (99% for synthesis) was purchased from Aldrich and distilled over CaH_2 prior each use. Thiourea (99+ for synthesis), 6-bromohexanoic acid (purum, for synthesis), phosphorus pentoxide (P_2O_5, 98%, synthesis grad), hydrochloric acid and potassium hydroxide were purchased from Aldrich Novozym®-435 (N435), acrylic resin-immobilized form of *Candida antarctica* lipase B (CALB), was purchased from Aldrich. Anhydrous toluene was freshly distilled over sodium under nitrogen atmosphere. Other solvents (GC grade) were purchased from Acros and used without further purification.

Synthesis:

6-mercaptohexanoic acid synthesis[3]:

6-bromohexanoic acid (70 mmol) was dissolved in ethanol (9.1 mL) and added dropwise to a hot (80-90 °C) and stirred solution of thiourea (70 mmol) in water (46 mL). Then, the mixture was stirred and heated to reflux (90-100 °C) for 2 hours. Then 30 mL of a KOH solution (8.3 $mol.L^{-1}$) were added and the reaction was allowed to proceed at 90-100 °C for 1 day. Medium was cooled down to room temperature, and then acidified with HCl solution (1

mol.L^{-1}) until pH=1. Mixture was extracted with chloroform and washed 3 times with water. Finally, solvent was stripped off by rotary evaporation at 35 °C under reduced pressure (40 mbar). A typical yield of 90% was obtained. The final product was characterized by NMR spectroscopy: ^{1}H NMR (400 MHz, $CDCl_3$): δ=1.35 ppm (t, 1H, *H*S-), δ =1.40-1.50 ppm (m, 2H, —C*H*$_2$—$CH_2CH_2CO_2H$), δ =1.57-1.70 ppm (m, 4H, —C*H*$_2$—CH_2—C*H*$_2$—CH_2CO_2H), δ =2.36 ppm (t, 2H, —C*H*$_2$—CO_2H), δ = 2.52 ppm (m, 2H, HS—C*H*$_2$—).

2.2.2 ε-thiocaprolactone synthesis [20]:

Typically, 27 mmol of 6-mercaptohexanoic acid (4 g) was placed in a 50 mL round bottom flask. 0.8 g of P_2O_5 was added. The flask was quickly fitted with a short distillation head. Then, the flask was immersed in an oil bath at 190 °C and pressure was lowered to 80 mbar. The distillation rate of thiolactone was maintained by lowering pressure gradually until 1 mbar. After 2 h, thiolactone was solubilized in ether and washed with aqueous $NaHCO_3$ until it reached neutral pH. Mixture was then dried over magnesium sulfate and ether was stripped off by rotary evaporation, giving typically a yield of 40%. The final product was characterized by ^{1}H NMR (400 MHz, $CDCl_3$): δ =1.70-1.90 ppm (m, 4H, —C*H*$_2$—CH_2—C*H*$_2$—CH_2COS), δ =2.05-2.15 ppm (m, 2H, $OSCH_2$—C*H*$_2$), δ =2.85 ppm (t, 2H, —C*H*$_2$—COS), δ = 3.05 ppm (t, 2H, COS—C*H*$_2$—).

One and two steps enzymatic ring opening polymerization:

All the homopolymerizations and the first series of copolymerization were carried out in dry toluene at 70 °C. For that, 4Å molecular sieve (0.1 g) was introduced into previously dried Schlenck tube under an inert dry argon atmosphere. Toluene and 6.9 mmol of the corresponding monomers (0.79 g and 0.90 g for ε-caprolactone and ε-thiocaprolactone, respectively and a mix of them in selected proportions) were transferred with a syringe through rubber septum caps. Predetermined amounts of catalysts, N435 (100 mg), were quickly introduced in the tube under an inert dry argon atmosphere. The tube was immediately capped with a rubber septum. The enzyme addition marked the beginning of the polymerization (t_0). After 72 h, reactions were stopped by dissolving the reaction mixture in chloroform and removing the catalyst and molecular sieve by filtration. The part of the solvent in the filtrate was then stripped by rotary evaporation at 35 °C and under reduced

pressure of 40 mbar. The polymer in the resulting concentrated solution was precipitated in cold methanol (previously stored at −20 °C) to ensure also the recovery of the shortest chains. The polymer precipitate was separated by filtration and dried overnight at 30 °C under vacuum.

For the two steps copolymerization setup we used similar procedures but the ε-caprolactone monomer was replaced by its equivalent weight of the previously synthesized PCL oligomers (8 kg.mol^{-1}).

Methods of characterizations

1D and 2D NMR spectra were recorded on a Bruker Ascend™ 400 spectrometer at 400 MHz, with, at least, 64 scan for ^{1}H and 100 MHz and 1024 scan for ^{13}C. $CDCl_3$ was used as solvent. ε-thiocaprolactone and ε-caprolactone conversions were determined (before precipitation) by the relative integration of peaks at 4.17 ppm (monomer, COO—C*H*$_2$—) and at 4.07 ppm (polymer, COO—C*H*$_2$—) for ε-caprolactone and peaks at 3.05 ppm (monomer, COS—C*H*$_2$—) and at 2.85 ppm (polymer, COS—C*H*$_2$—) for ε-thiocaprolactone. Thiolactone units content in final polymers (recovered after precipitation) was determined using the equation (1) by integration of the peaks at 4.07ppm for poly(ε-caprolactone) ($I_{poly(\varepsilon-caprolactone)}$) and at 2.85ppm for poly(ε-thiocaprolactone ($I_{poly(\varepsilon-thiocaprolactone)}$).

$$\varepsilon - thiocaprolactone\ content\ (\%) = \frac{I_{poly(\varepsilon-thiocaprolactone)}}{I_{poly(\varepsilon-thiocaprolactone)}+I_{Poly(\varepsilon-caprolactone)}} \times 100 \qquad (1)$$

Size exclusion chromatography (SEC) measurements were performed in chloroform (HPLC grade) in a Shimadzu liquid chromatograph equipped with a LC-10AD isocratic pump, a DGU-14A degasser, a SIL-10AD automated injector, a CTO-10A thermostated oven with a 5 µm PLGel Guard column, two PL-gel 5 µm MIXED-C and a 5 µ 100 Å 300 mm-columns, and three online detectors: a Shimadzu RID-10A refractive index detector, a Wyatt Technologies MiniDAWN 3-angle-light scattering detector and a Shimadzu SPD-M10A diode array (UV) detector. Molar masses and dispersity were calculated from a calibration with polystyrene standards in UV or RI detection. Samples were dissolved in chloroform (concentration 4 mg.mL^{-1}) and filtered through a 0.45 µm PTFE membrane. For all analyses the injection volume was 100 µL, the flow rate was 0.8 mL.min^{-1} and the oven temperature was set at 25 °C.

For MALDI-ToF MS analysis, samples were prepared using a freshly prepared matrix solution of Super DHB (9:1 mixture of 2,5-dihydroxybenzoic acid and 2-hydroxy-5-methoxybenzoic acid, from Sigma Aldrich) dissolved to saturation in a H_2O/CH_3CN + HCOOH (50/50 + 1%) solution. Typically, a 1:1 mixture of the sample solution in CH_2Cl_2 was mixed with the matrix solution and 1 µL of the resulting mixture was deposited on the stainless steel plate. Mass spectra were acquired on a time-of-flight mass spectrometer (MALDI-ToF-ToF Autoflex II ToF-ToF, Bruker Daltonics, Bremen, Germany) equipped with a nitrogen laser (λ = 337 nm). An external multi-point calibration was carried out before each measurement using the singly charged peaks of a standard peptide mixture (0.4 µM, in water acidified with 1% HCOOH). Scan accumulation and data processing were performed with FlexAnalysis 3.0 software.

The thermal stability and the degradation of the polymers were investigated by thermogravimetric analysis (TGA) coupled with a FTIR for evolved gas analysis. TGA measurements were conducted under dry air (at a flow rate of 75 $mL.min^{-1}$) using a Hi-Res TGA Q5000 apparatus from TA Instruments. The samples (5-9 mg placed in a platinum pan) were heated up to 450 °C at 5 $°C.min^{-1}$. FTIR spectra were recorded on a Nicolet 380 (Thermo Electron Corporation) by performing 16 scans with 4 cm^{-1} resolution.

Differential scanning calorimetry (DSC) measurement were performed on a TA Q200 DSC in sealed aluminum pan, typically on about 1 mg of purified sample, in a heat-cool-heat curse at 10 $°C.min^{-1}$ for heating and 5 $°C.min^{-1}$ for cooling. Results were exploited using TA Universal Analysis software. To determine the crystallinity content, we used 163.3 $J.g^{-1}$ determined from Joback's methodology [34] as an approximation of ΔH_m^0 for pure ε-thiocaprolactone. For neat PCL, the common value of 139.3 $J.g^{-1}$ was used [35]. For copolymers we used an average value based on monomers composition.

Results and Discussions

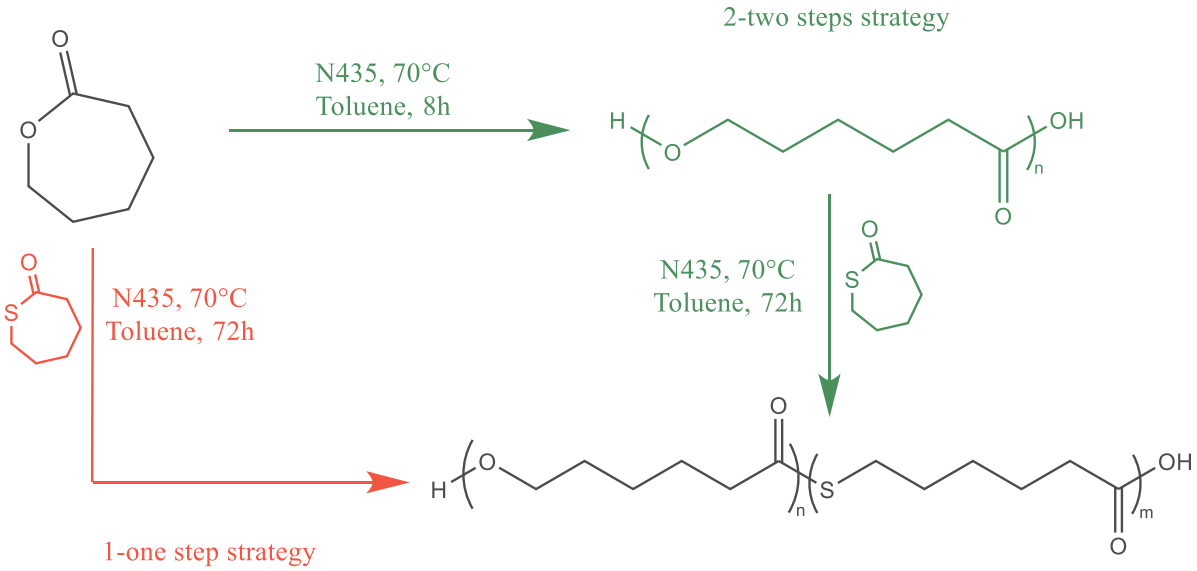

***Figure IV-2**. ε-caprolactone and ε-thiocaprolactone copolymerization strategies*

Table IV-1. Main results of the homo and copolymerization of ε-thiocaprolactone and ε-caprolactone catalyzed by N435, with various molar ratios

Entry N°	ε-thiocaprolactone feed content (mol%)	Final ξ (%)[a]	Polymer η (%)[b]	Mn[c]	Đ[c]	$T_{d\text{-}10\%}$[d] (°C)	$T_{d\text{-}Max}$[e] (°C)	T_m (°C)[f]	ε-thiocaprolactone polymer content (mol%)[g]	*X* (%)[h]
1	**0**	**>98**	**95**	**15,000**	**1.7**	**292**	**341**	**61**	**0**	**59**
2	25	83	80	10,800	1.7	294	362	56	14	56
3	50	70	65	8,600	1.9	325	400	43	27	27
4	75	72	69	4,100	1.3	321	399	50	61	47
5	**100**	**77**	**70**	**4,400**	**1.1**	**322**	**379**	**55**	**100**	**53**
6	25	85	79	5,000	1.6	290	351	43-57	16	n.d.
7	50	68	61	2,900	1.5	335	370	31-46	19	n.d.
8	75	76	69	3,100	1.4	336	374	46-57	55	n.d.

a: Yield determined by NMR before precipitation; b: Yield determined by weighing after precipitation and drying; c: Molar mass (g/mol) determined by SEC; d: Temperature at 10% weight loss determined by TGA; e: Temperature at maximum of degradation determined by TGA; f: Melting temperature determined by DSC; g: Fraction determined by NMR before precipitation; h: Crystallinity content

approximated using Joback/Reid methodology [34]; n.d.: Not determined (cannot be precisely determined due to peaks overlapping).

General strategies:

To synthesize poly(ε-caprolactone)-*co*-(ε-thiocaprolactone) with controlled structures and to understand the effect of some parameters, two copolymerization strategies have been performed (Figure 2). These strategies are based on a one step (one pot) or a two steps procedure. For this latter, a first step is based on the synthesis of PCL chains and then after the addition into the medium of ε-thiocaprolactone, in a second step, to synthesize the copolyesters (Figure 2). All formed copolymers have been characterized by several analytical methods and the results are summarized in Table 1. Entries N° 2, 3 and 4 correspond to the 1-step copolymerization. Entries N° 6, 7 and 8 correspond to the 2-steps copolymerization procedure. Entries N°1 and 5 are linked to the synthesized homopolymers, PCL and PTCL, respectively. NMR characterization of those homopolymers could be found in supporting information (SI) Figure SI1 for PCL and SI2 for PTCL. Compared to neat PCL; we can notice that neat PTCL presents a limited molar mass and a higher degradation temperature on agreement with previous publications [11]. However, Tm and crystallinity content are closed for both homopolymers.

One step copolymerization:

This way is based on the polymerization of a mixture of both monomers Such procedure could give us information on the relative reactivity of the two monomers with the enzyme. From table 1, we can notice that we did not reach the initial ratio introduced in the medium due to the uncomplete integration of ε-thiocaprolactone monomers into the macromolecular architectures. These unreacted monomer content induces decreased yields. For instance, in N°2, only 14% of thiomonomers are inserted compared to the 25% targeted content (corresponding gap = 11%) and we noticed a yield of only 84%. This gap increases with decreasing yields (N°3-4).

Table 1 shows a decrease in molar mass with the increase in ε-thiocaprolactone content (from 15,000 g.mol^{-1} for neat PCL to about 4,000 g.mol^{-1} for neat PTCL). As it can be seen from Figure SI3 in SI, this decrease seems to be linear until reaching a plateau at about 4,000

g.mol^{-1} for about 55 percent of ε-thiocaprolactone inserted. Such a behavior is not very surprising due to the difference of reactivity and since thiol function, although close to hydroxyl function, is not part of natural substrates of lipases and could be less adequate as enzymes binding site. Indeed, Weber et al. performed lipases catalyzed thioesterification on model fatty thiol and fatty acids[32] and observed limited conversion while we observed quantitative conversion for esterification of similar compound (fatty alcohol with fatty acid)[37]. In similar conditions Matsumura and coworkers[3] have similar yield but the decrease of molar mass is not so marked in their work, with a minimum molar mass reached for 82% of ε-thiocaprolactone and greater molar mass for neat PTCL.

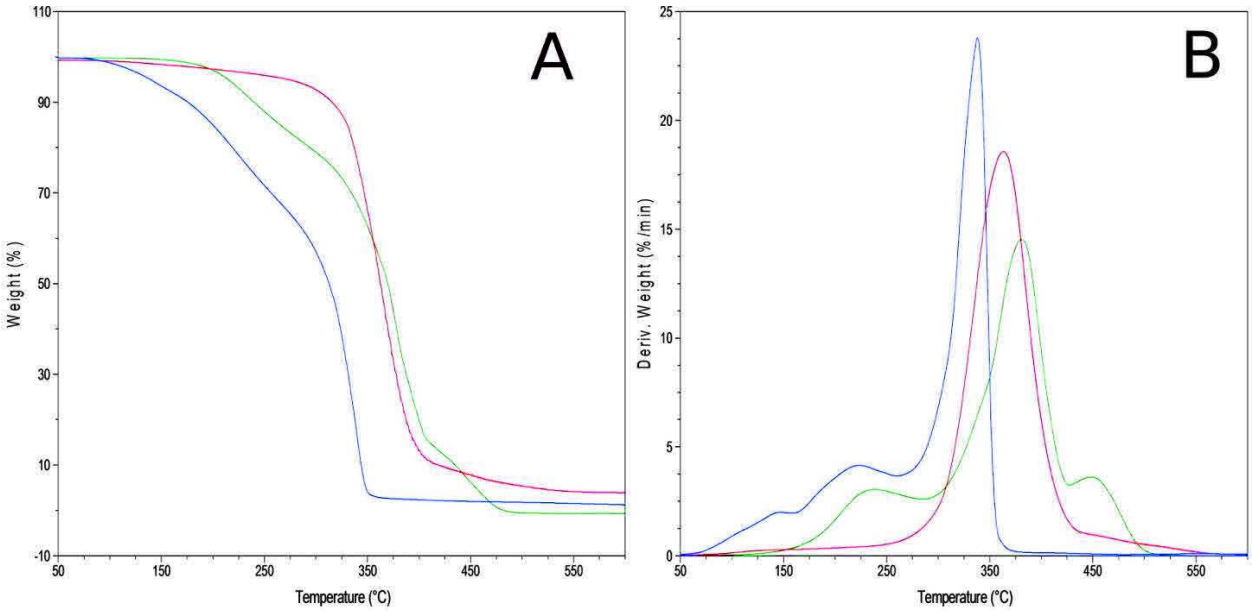

***Figure IV-3**. Degradation profiles (ATG (A) and DTG (B)) of PCL in red, PTCL in green and for the copolymer containing 14% of TCL synthesized by one step strategy in blue.*

The profile of the thermal behavior characterization of copolymers from the one-step copolymerization profiles are given on Figure 3 (for both PCL and PTCL homopolymers and for a copolymer containing 25 percent of TCL). The DTG profiles show that PCL and PTCL show multistep degradation processes. The first degradation step at about 250°C for PCL and 280°C for PTCL corresponds to the loss of the residual monomers and small oligomers. PTCL shows a last degradation step at around 450°C due to ashes formation but this specific point need further investigations. The starting of the degradation is a key parameter since it gives the highest temperature for the conventional usage of the material. Figure 3 clearly shows

that PCL starts to degrade at a temperature largely lower tan PTCL. Table 1 shows an increase of degradation at 10% mass loss at about 292 and 322 °C for neat PCL and PTCL, respectively. $T_{d\text{-}10\%}$ seems to display a maximum value (N°3) and a plateau around 320 °C (Fig. SI4 in SI). Similar behavior is observed with the maximum degradation ($T_{d\text{-}Max}$) temperature with a plateau at around 400°C. Such a behavior is expected because of the higher thermal stability of thioesters and sulfur compared to the polyesters [22].

One can also notice that the melting temperatures of our copolymers are significantly lower than those reported in the literature for copolymer with different monomer ratios [3] or for PTCL (around 105 °C in the literature)[2]. Contrary to the results and trends reported in the literature for this kind of copolymers[3] we noticed a decrease in melting temperature with the increase of ε-thiocaprolactone content. However, this could be explained by the lower molar mass we obtained (from 25% to 50% lower) compared to those obtained by Matsumura et al. [3].

Interestingly, both the melting temperature and crystallinity show a minimum value for 29% of thiomonomer content (Fig. SI4 and Fig. SI5 in SI) which is lower than the values measured for the corresponding homopolymers. As previously stated, such a trend is not surprising because of the relative chemical similarity between thioesters and esters functions. The slightly lower esters-thioesters interactions, compared to esters-esters and thioesters-thioesters interactions, may explain the lower crystallinity and melting temperature for the “medium” compositions compared to the compositions based on higher monomer contents.

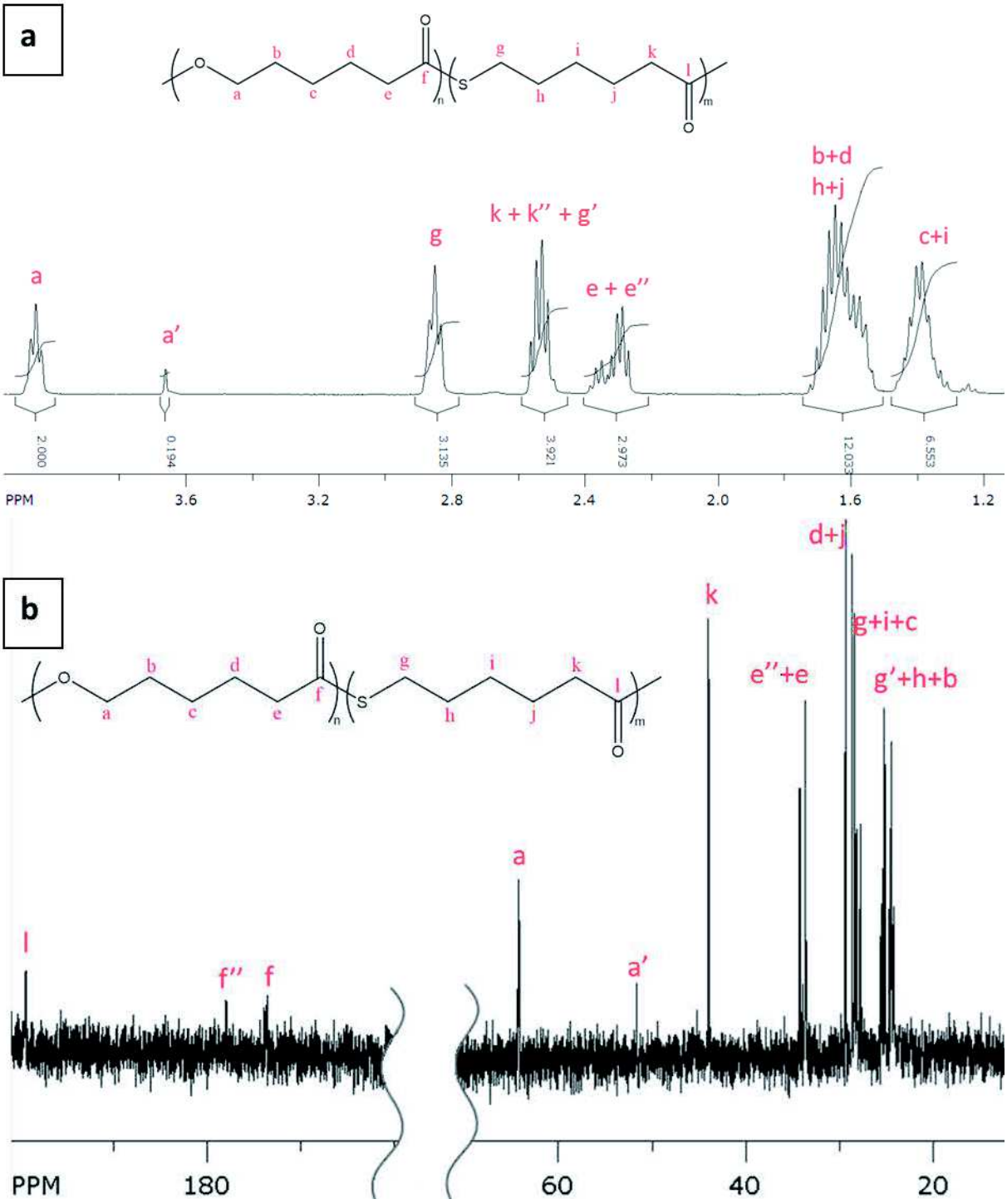

Figure IV-4. *1H (a) and 13C NMR (b) assignments for the copolymer N°4 with 61% ε-thiocaprolactone content (prime corresponding to the hydroxyl or thiol chain ends, and double prime corresponding to the acid chain ends).*

From the NMR characterization of copolymers synthesized by the one-step procedure (Fig. 4), one can notice there are numerous small peaks on ^{13}C analysis (Fig. 4b), between 24 and

28 ppm (corresponding to hydrocarbon chains between ester or thioester functions), which are linked to different monomers environments and sequences, but that cannot be assigned more precisely by this method.

Generally, these assignments are in accordance with those reported in the literature, but we are not able to see the CH_2SH peak neither in proton nor in carbon spectra. However, its presence was confirmed by HSQC 2D NMR (spots for directly linked proton and carbon) experiment that shows a correlation spot indicating its presence under the multiplet at 2.51-2.56 ppm on the proton spectrum and around 28 ppm on carbon spectrum (Fig. 5).

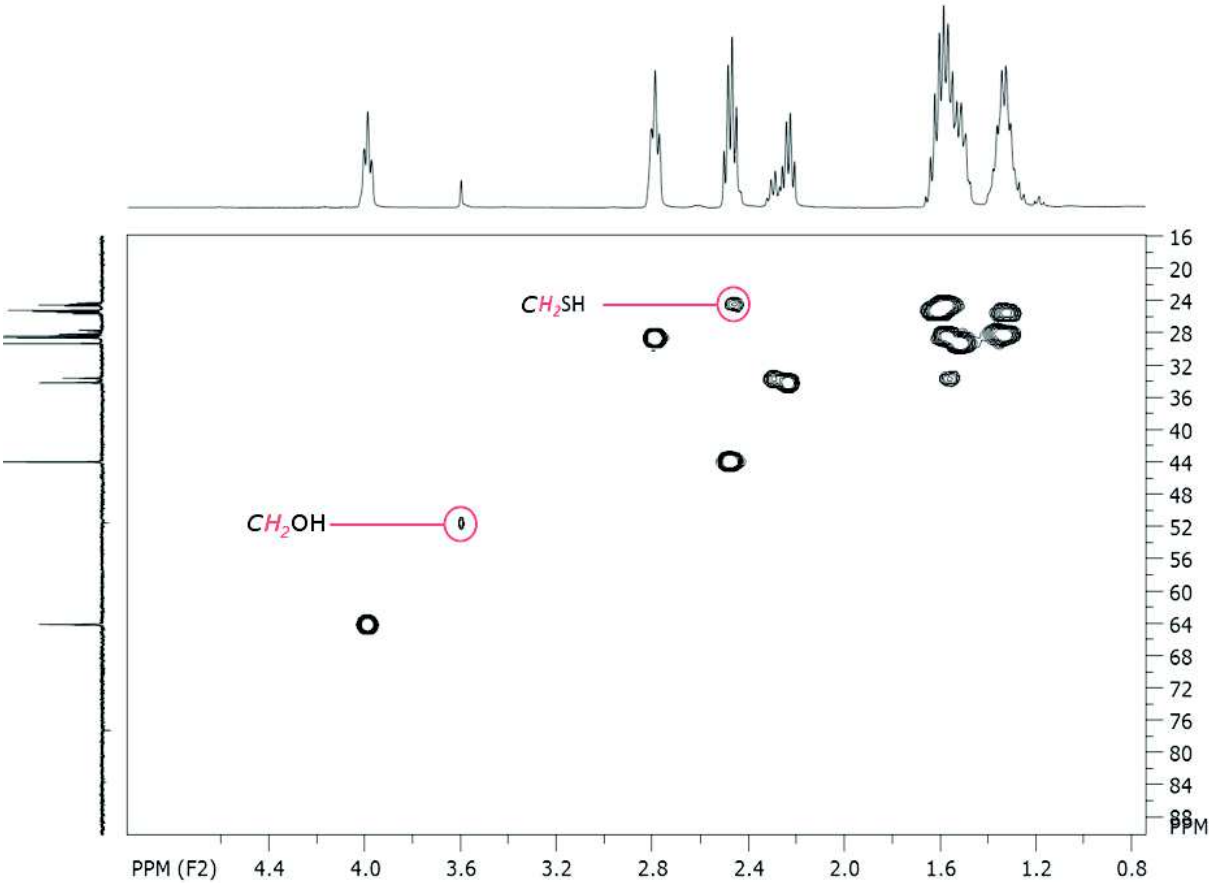

Figure IV-5. *HSQC 2D NMR spectrum of copolymer with 61% of ε-TCL*

The relative intensity of the spot corresponding to *CH_2*SH (25 ppm x 2.5 ppm) compared to the spot corresponding to the *CH_2*OH (52 ppm x 3.6 ppm) seems to indicate that there are more SH terminated chains than OH terminated ones (spectra for copolymers N°2 and 3 are available in supporting information, Fig. SI6 to SI7). This could be due to the fact that thiolactone addition may lead to more termination reactions. As a result, unfavorable

interaction of thiol-terminated oligomers in the enzyme binding site could favor the expulsion of the growing chain out of the catalyst site. This point is also in accordance with the uncomplete TCL conversion and it could explain the decrease of the molar mass with the increase of the thiolactone monomer content. The other signals on HSQC spectrum are in accordance with the previous ^{1}H and ^{13}C NMR assignments.

For the copolymers obtained from the one-step procedure, all the MALDI-ToF spectra look like the typical one shown in Figure 6. The distributions are monomodal and centered at m/z 6,400, 4,200 and 4,000 for the copolymers having 14%, 29% and 61% of TCL, respectively. For each peak of the distribution, we could find the different potential monomers compositions. From the NMR results (see Table 1) we could determine the probable monomers composition for each peak. For example, for the spectrum of the copolymer with 14% TCL content (Fig. 6), the peak at 5,506 m/z likely corresponds to a chain with 7 TCL and 40 lactone units (which represents 15 mol% of thiolactone content) and the peak at 6,696 m/z likely corresponds to 10 TCL and 47 CL units (which represents about 18 mol% of TCL in the chain). Chain-ends of all polymers are based on H and OH groups indicating that the polymerization reaction was initiated by water molecules. As expected, the interval between two successive peaks of the homopolymers distribution corresponds to one CL unit (+114 m/z) for pure PCL and to one TCL unit (+130 m/z) for neat PTCL.

Interestingly, one can notice that for copolymers such an interval is around +48 m/z on agreement with Equation 2, where M is the molar mass (g/mol) and P is the peak (m/z).

$$P_i = P_{i-1} + 3(M_{Thio} - M_{CL}) \quad (2)$$

This very uncommon distribution, with a mass interval corresponding to the difference between a PCL trimer and a PTCL trimer, could be due to a specific multistep addition mechanism. Indeed, this distribution could be explained by the addition of monomers three by three for TCL and for CL, which likely means that there is a prior step of trimerization before the polymerization itself. Even if it was not expected, this is not so surprising since it is known that CALB is able to synthesize di- and tri-(ε-thiocaprolactone). In some works, these oligomers represent about 30% of the products [31]. Finally, this particular distribution is possible if we assume that there is a two steps mechanism: in a first step CL and TCL are respectively oligomerized to form trimers which could be cyclic or not, and in a second step we have the polymerization of these trimers.

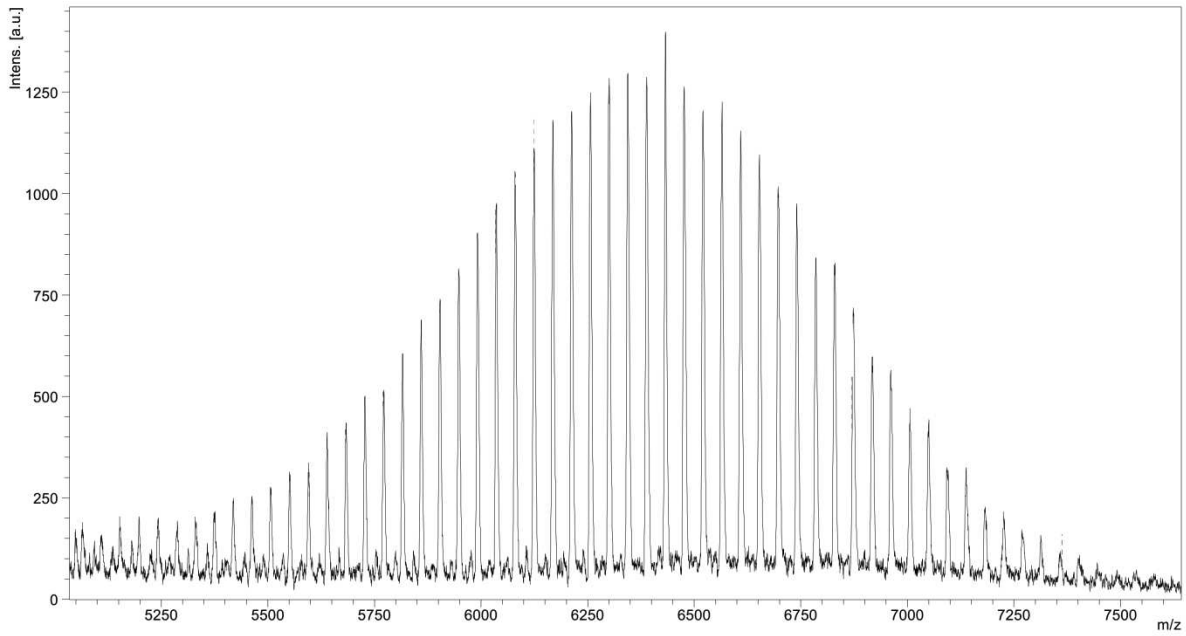

Figure IV-6. *MALDI-ToF spectrum of copolymer with 86% of ε-caprolactone and 14% of ε-thiocaprolactone*

Two steps copolymerization:

The second strategy is based on a two-steps procedure aimed to produce some structured copolymers based on CL and TCL (Fig. 2). Some of us reported the beneficial use of such a strategy for the eROP of lactide starting from previously formed PCL chains[38]. Thus, we started with enzymatically synthesized oligo(ε-CL) of 8,000 g.mol^{-1} from which we tried in a second step to polymerize TCL units.

Table 1 (N° 6-8) shows a significant conversion of TCL monomer. The yields are comparable to those obtained with the first strategy, which tend to confirm that the yield loss is probably due to incomplete polymerization of TCL monomers. However, one can also notice a significant lowering of final molar mass compared to the initial Mn of PCL oligomers from the first step. Such a decrease in the second step could be explained by the occurrence of numerous transesterification reactions which are catalyzed and significantly promoted by N435 [36].

NMR spectra of copolymers obtained from the two steps strategy and given in the SI (Fig. SI8 to SI10 for copolyesters N°6 to 8) are very similar to those of our first copolymerization

setup. In fact, this technique does not allow us to determine if there is a specific macromolecular architecture or sequence of monomer units due to the significant chemical differences between the monomers. The most characteristic signal should be the coupling spot between protons on S-CO-CH_2 and CH_2-O-CO that may be visible only in HMBC (Heteronuclear multiple-bond correlation, ^{1}H-^{13}C dipolar coupling) experiment, but these protons are too distant and in too low proportion in the copolymers to give a visible spot.

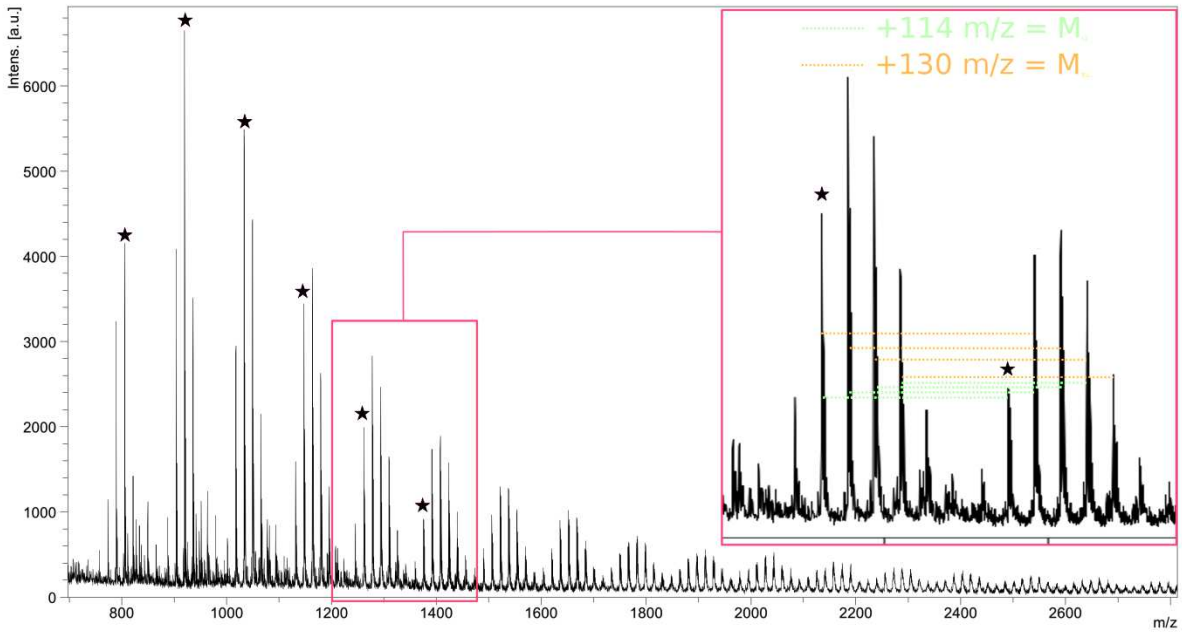

Figure IV-7. *MALDI-ToF spectrum of copolymers (55% ε-thiocaprolactone content) synthesized by the two steps strategy, stars mark peaks corresponding to PCL homopolymer distribution*

Maldi ToF spectra of poly(CL-co-TCL) from the two steps strategy (N° 6-8, Table 1) show a m/z distribution split in small peaks groups (Fig. 7). Two successive groups have their main peaks separated alternatively by 114 m/z and 130 m/z, which correspond to one CL and to one TCL, respectively (Figure 7 inset). Each group shows a small distribution in which the successive peaks are separated by m/z of 16, corresponding to the mass difference between one CL and one TCL. This kind of distribution is compatible with a ε-thiocaprolactone monomer opening and insertion occurring from split PCL chain as initiator. At least, those significant differences between spectra of the two polymerization setup suggest different copolymers structuration.

It seems that the copolymers produced by the two steps copolymerization do not show a mechanism of trimers addition contrary to those from the first strategy. However, MALDI-

ToF spectra do not allow us to conclude on the absence of such a mechanism, since the use of preformed PCL chains as initiator could hide this phenomenon.

Table 1 present also the main ATG results. We can notice the same trends as for the one step procedure. For close TCL content, $T_{d\text{-}10\%}$ gaps between one or two steps synthesis are low (Table 1). For instance, $T_{d\text{-}10\%}$ is 294 °C for N° 2 (one-step procedure, 14% TCL versus 290 °C for N° 6 (two steps procedure, 16% TCL). For the same copolyesters at the maximum degradations we can notice 362 and 351°C, respectively, for $T_{d\text{-}Max}$.

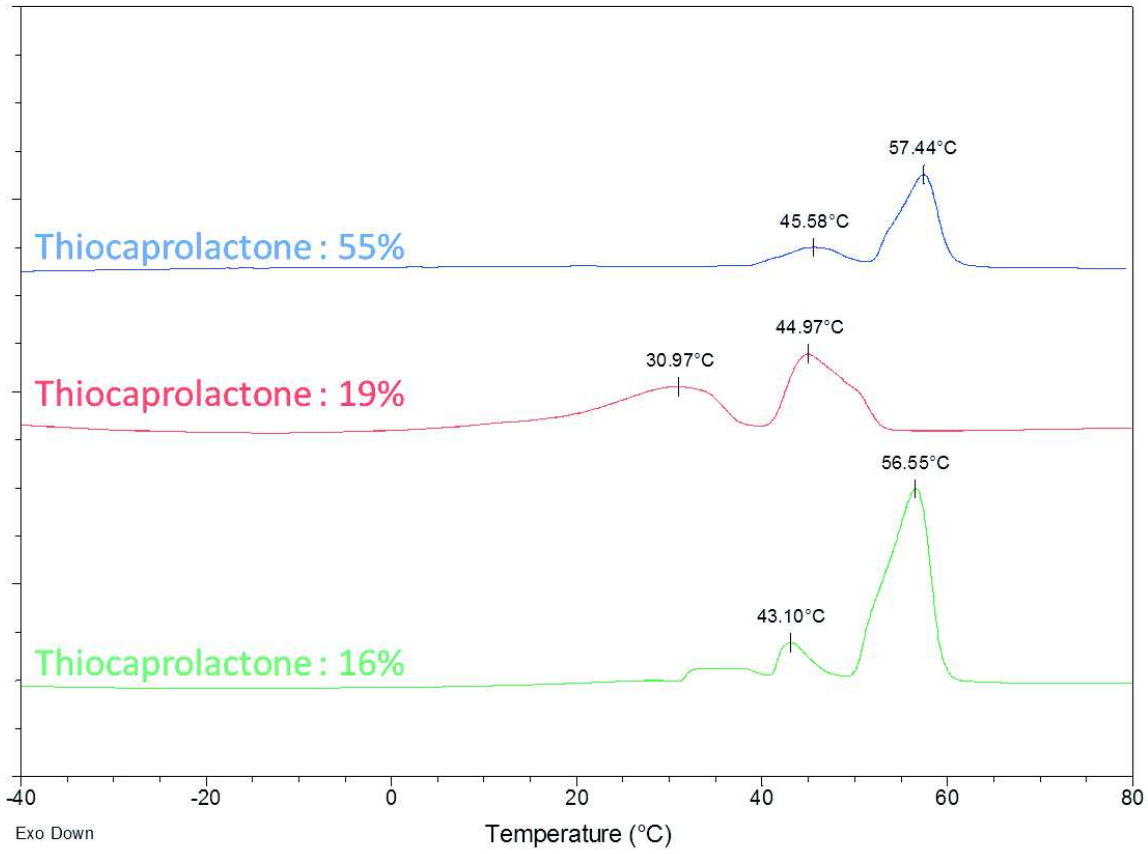

***Figure IV-8**. DSC thermograms of copolymers synthesized by the two steps strategy*

With this second strategy we aimed at producing blocky structured copolymers using preformed CL oligomers as initiator for TCL eROP. Blocky structures could result in some phase separation between the thioester rich segments and the ester rich segments which could be observed by a multiple melting peak in DSC thermogram. Interestingly, all products from the two steps strategy (N° 6-8) show one crystallization peak and two melting temperatures (Fig. 8), which could be due to the presence of distinct domains in the formed oligomers. As previously mentioned, this is likely due to some segments rich in CL units

which probably show melting temperature around 50 °C (59 °C for neat PCL, Table 1) and segments enriched in TCL units which show T_m around 40 °C (to be compared with 53 °C measured for neat PTCL and 43 °C for the 27 mol% of TCL, Table 1). The structures of these copolymers with two parts, one enriched in CL units and one enriched in TCL units, could be explained by a growing mechanism involving in a first step PCL chain splitting followed by TCL addition and growing from these split PCL chains acting as initiators.

There are two melting peaks spaced by 14 °C for the copolymer composition with 16% in TCL units, 11 °C for the 55% in TCL units and 14 °C for the 19% in TCL units (Table 1, N° 6-8) that corroborate our hypothesis of a blocky structure. This is also in agreement with the mass spectroscopy results. However, only oligomers were obtained by this two steps strategy, then we cannot affirm that such a structure will not be balanced by transesterification and trans thio-esterification reactions if larger chains are obtained because of the well-known ability of N435 to catalyze transesterification especially for longtime reactions. Furthermore, it could be noticed that the smallest oligomers were produced for the equimolar feed composition (N° 7) and this could explain the lower melting temperature observed for this reaction.

Figure 8 shows that the relative melting enthalpy of both peaks (possibly corresponding to thioester-rich segments for the lower and ester-rich segments for the higher) seems to be independent of the monomers ratio. The lower temperature melting peak (around 45 °C for the 55 mol% and the 16 mol% TCL content) seems to be due to lower crystalline segments compared to the highest one (around 57 °C for the 55 mol% and the 16 mol% TCL content). This result suggests that there are segments (more crystalline) composed of numerous CL units with few incorporated TCL units. They result from transesterification reactions between TCL and the preformed PCL chains, and others segments (less crystalline, with lower melting point) likely resulting from the copolymerization of TCL unit with oligo-CL released in the medium by the aforementioned transesterification reactions. To conclude

Conclusions

The successful synthesis and complete chemical characterization of new copolymers based on ε-caprolactone and ε-thiocaprolactone by enzymatic ring opening polymerization has been fully reported. Thanks to the two different copolymerization strategies and numerous

advanced analyses we have been able to highlight a specific polymerization mechanism of trimers addition which had never been reported so far in eROP.

From the first strategy, poly(ester-co-thioester) has been synthesized with particular thermal properties, with a lower Tm than expected, and a very peculiar polymerization mechanism with a first step of trimerization before the polymerization of formed trimers.

The two steps polymerization strategy offers the possibility to synthesize copolymers by eROP of lactone and thiolactone with some control of the final architectures. This strategy allows obtaining copolymers with a probable blocky structure but with limited chain lengths. This strategy could be further improved by starting from larger preformed chain and by optimization of the thiolactone polymerization conditions.

We have shown that the molar mass of TCL-based polymer and its copolymers with CL have a huge influence on thermal properties. This interesting behavior could lead to polymers with tunable thermal properties with a melting temperature that could be varied from more than 100 °C for high TCL content and high molar mass polymers to about 40 °C for 27 mol% of TCL and low molar mass polymers.

We have demonstrated the possibility to produce, by enzymatic ring opening polymerization, copolymers of lactone and thiolactone with a blocky structure. Then this result paves the way for new interesting perspectives for the synthesis of polymers with well-defined and controlled architectures by playing on different reaction parameters such as the length, structure and functionality of the initiator, for example. This work also opens new investigation topics both on the mechanistic aspect of the reaction and on the synthesis of new biomaterials for different green and biomedical applications.

Suporting Informations

of "Enzymatic synthesis of poly(ε-caprolactone -co- ε-thiocaprolactone)"

containing supplementary NMR and MALDI-ToF caracterizations au synthezised copolymers. Evolution of molar mass, melting and degradation temperatures, crystallinity content as function of ε-thiocaprolactone content.

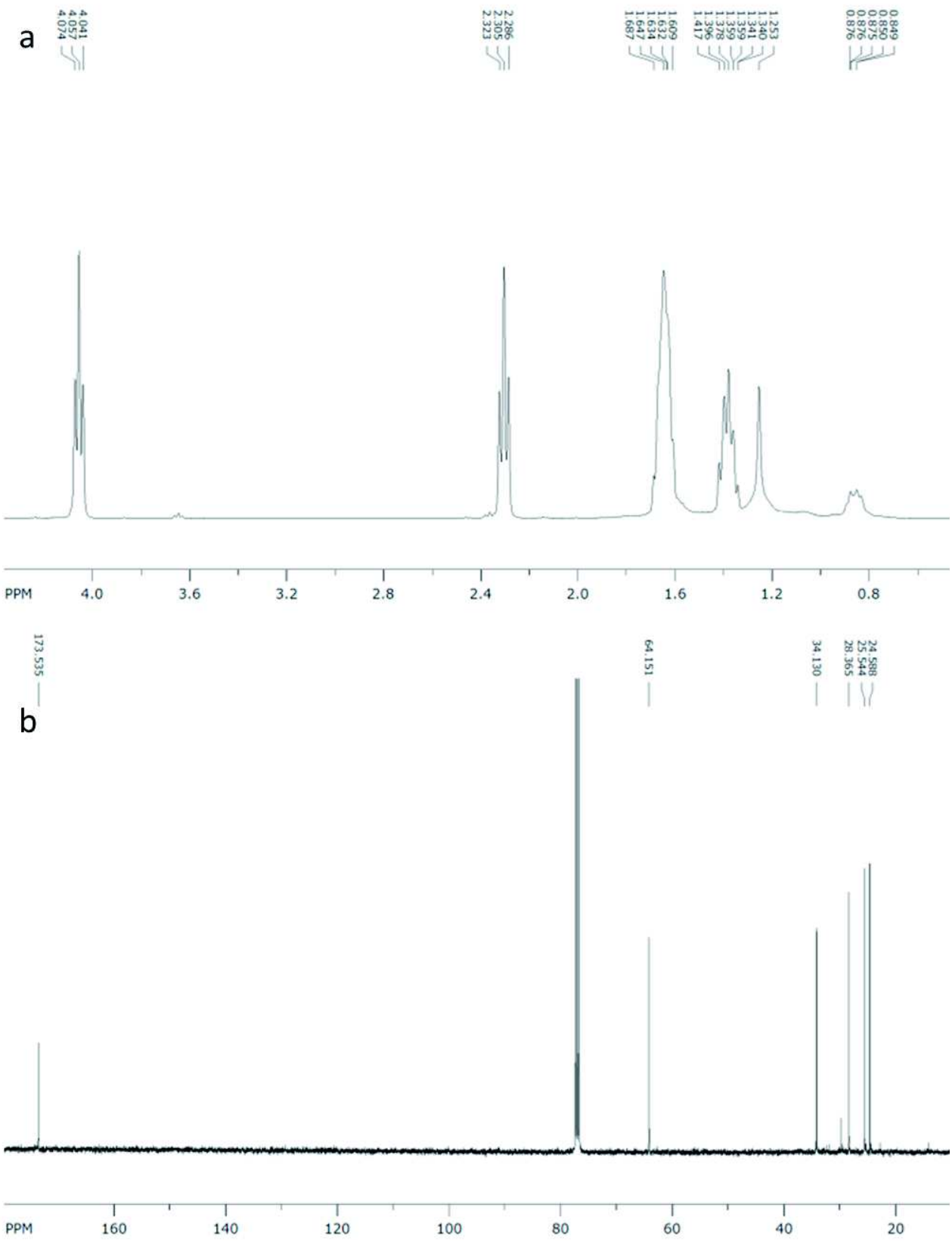

Figure SI IV-1. poly(ε-caprolactone), a 1H NMR spectrum, b 13C NMR spectrum

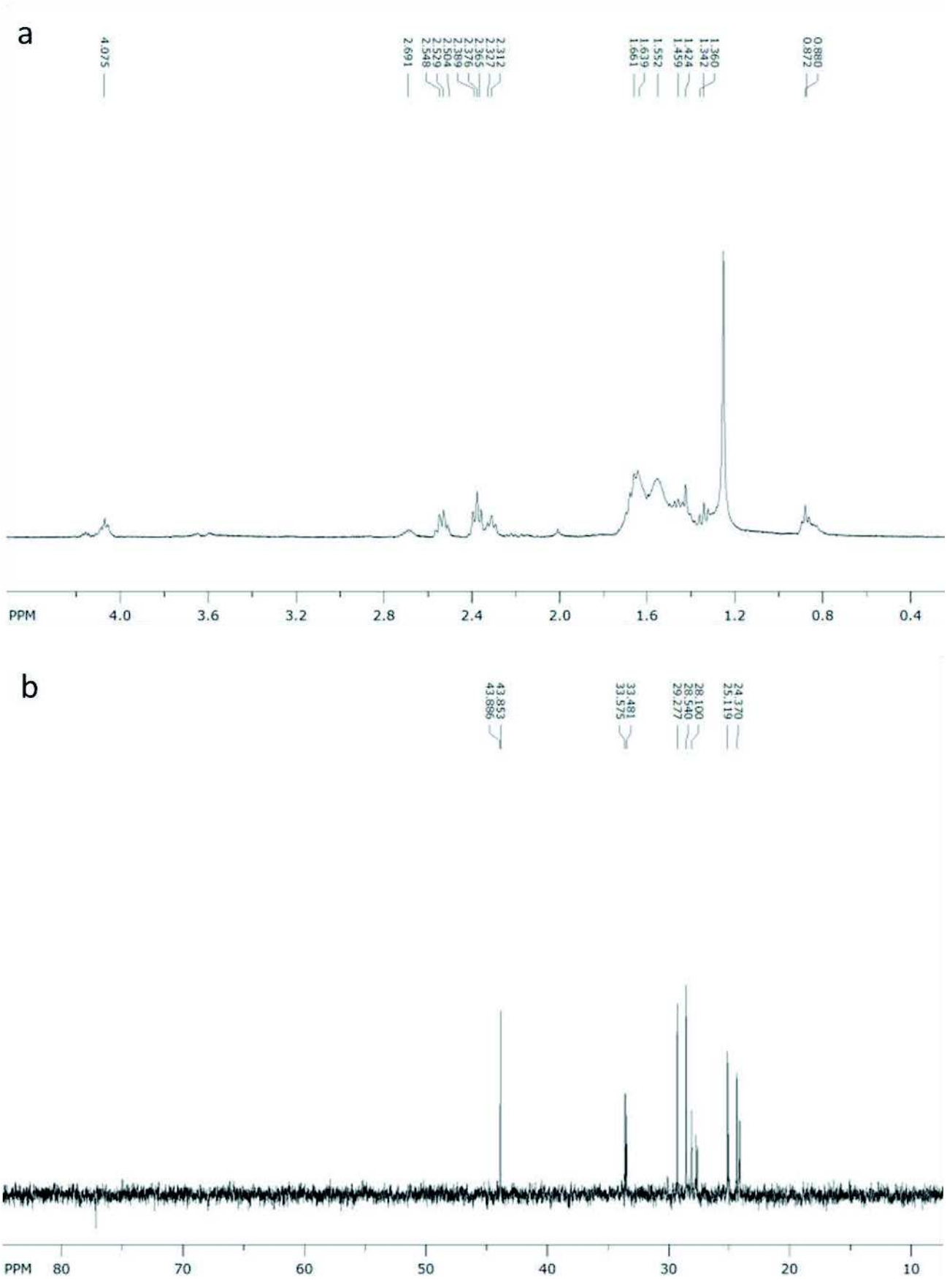

Figure SI IV-2. poly(ε-thiocaprolactone), a 1H NMR spectrum, b 13C DEPT NMR spectrum

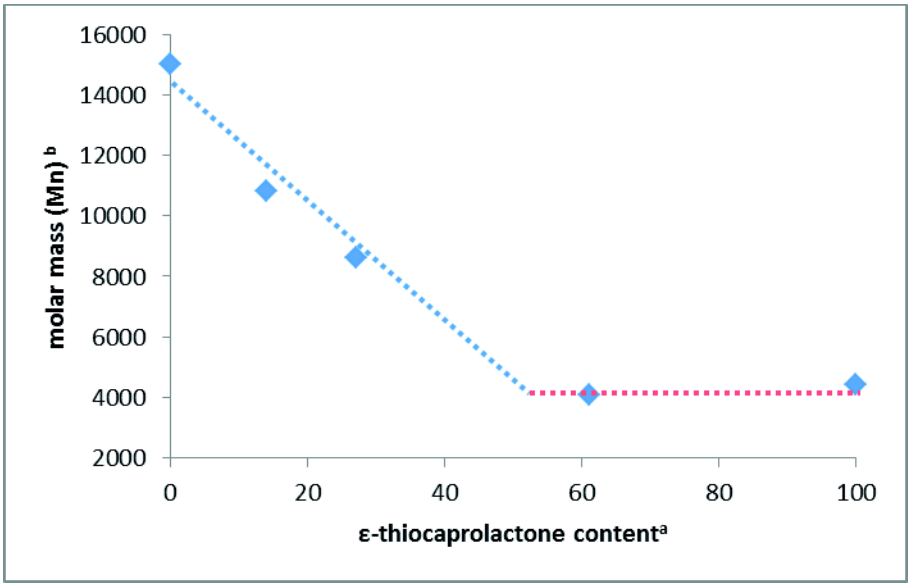

Figure SI IV-3. evolution of molar mass (Mn) as function of ε-thiocaprolactone content (a: determined by NMR before precipitation; b: determined by SEC).

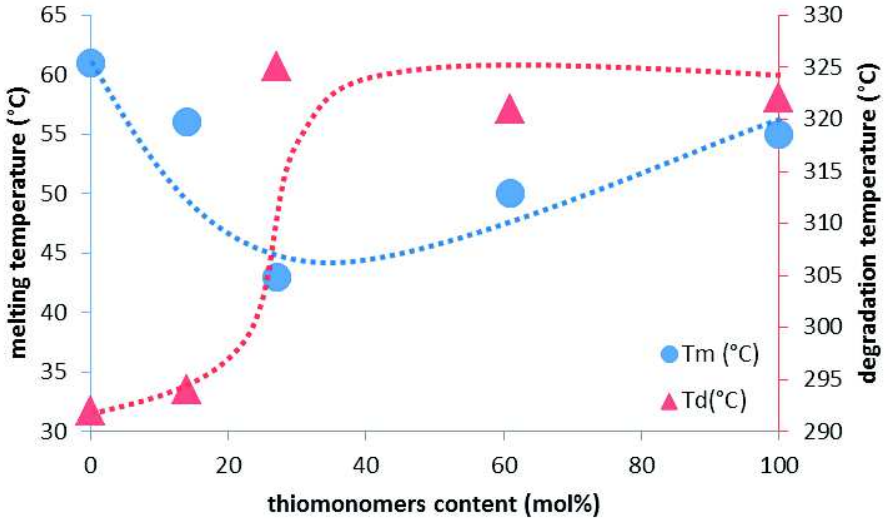

Figure SI IV-4. evolution of melting temperature (●) and degradation temperature (10% weight loss) (▲) as a function of ε-thiocaprolactone content

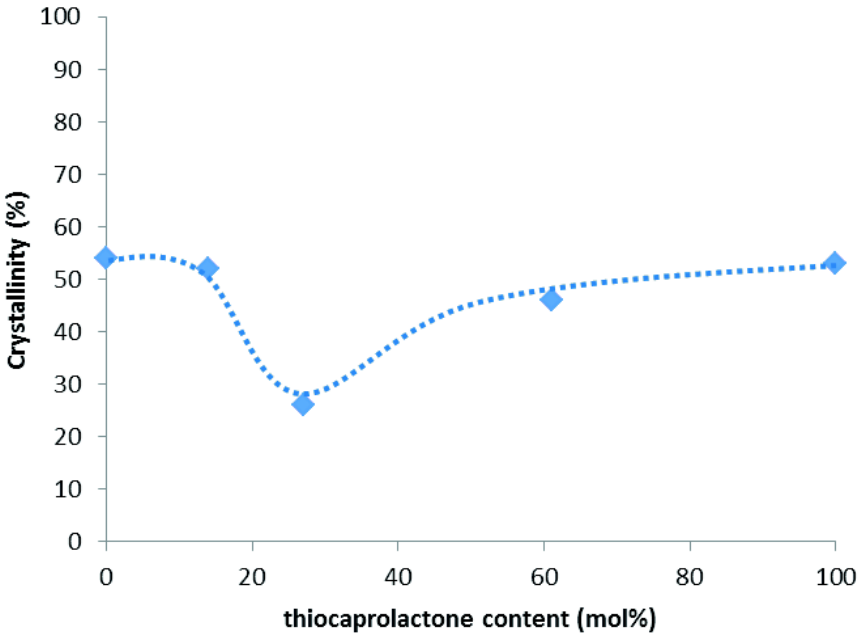

Figure SI IV-5. Crystallinity content as function of ε-thiocaprolactone content

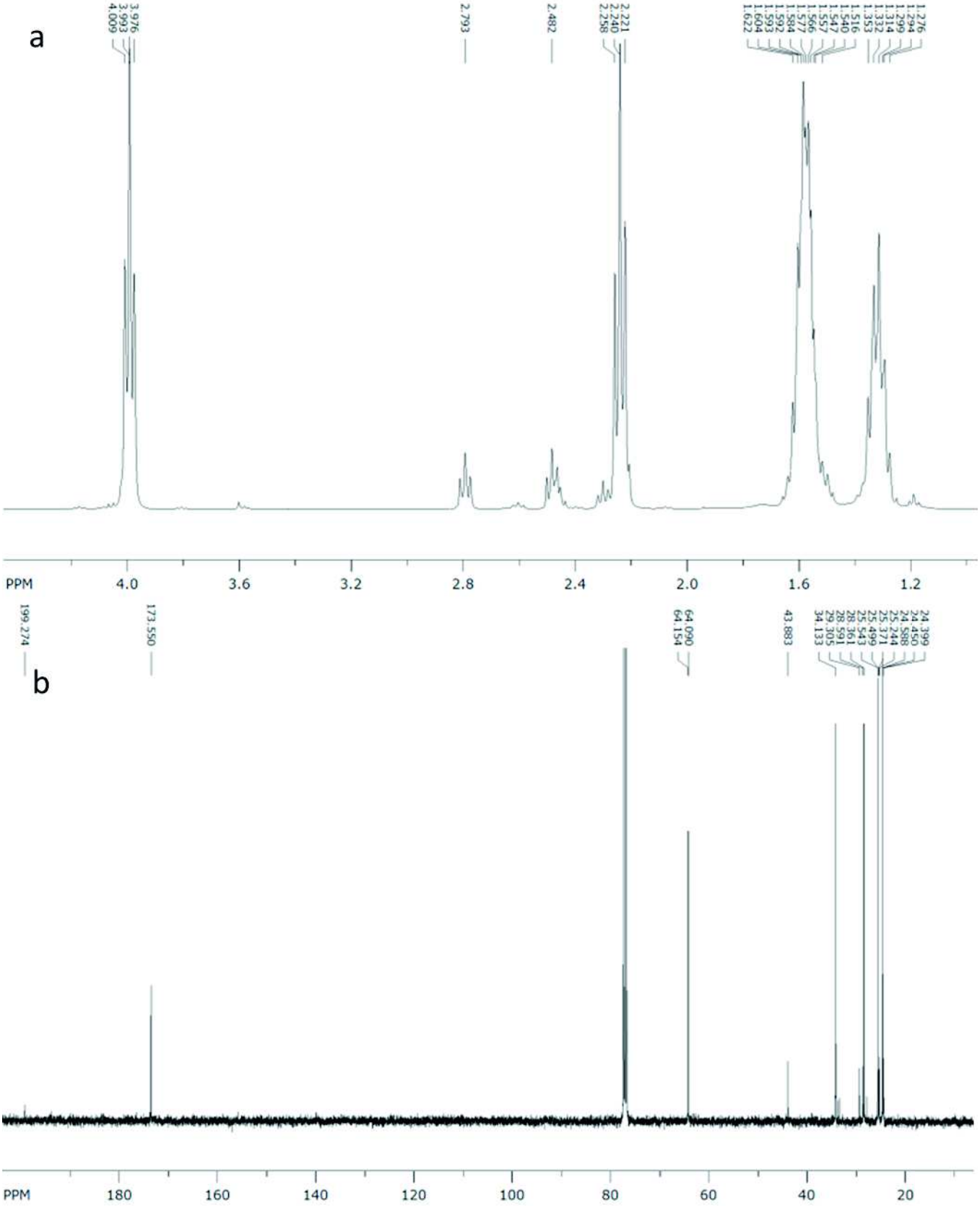

Figure SI IV-6. copolymers N°2 (14% ε-thiocaprolactone content) synthesized by the one step strategy; a- 1H NMR spectrum, b- 13C NMR spectrum

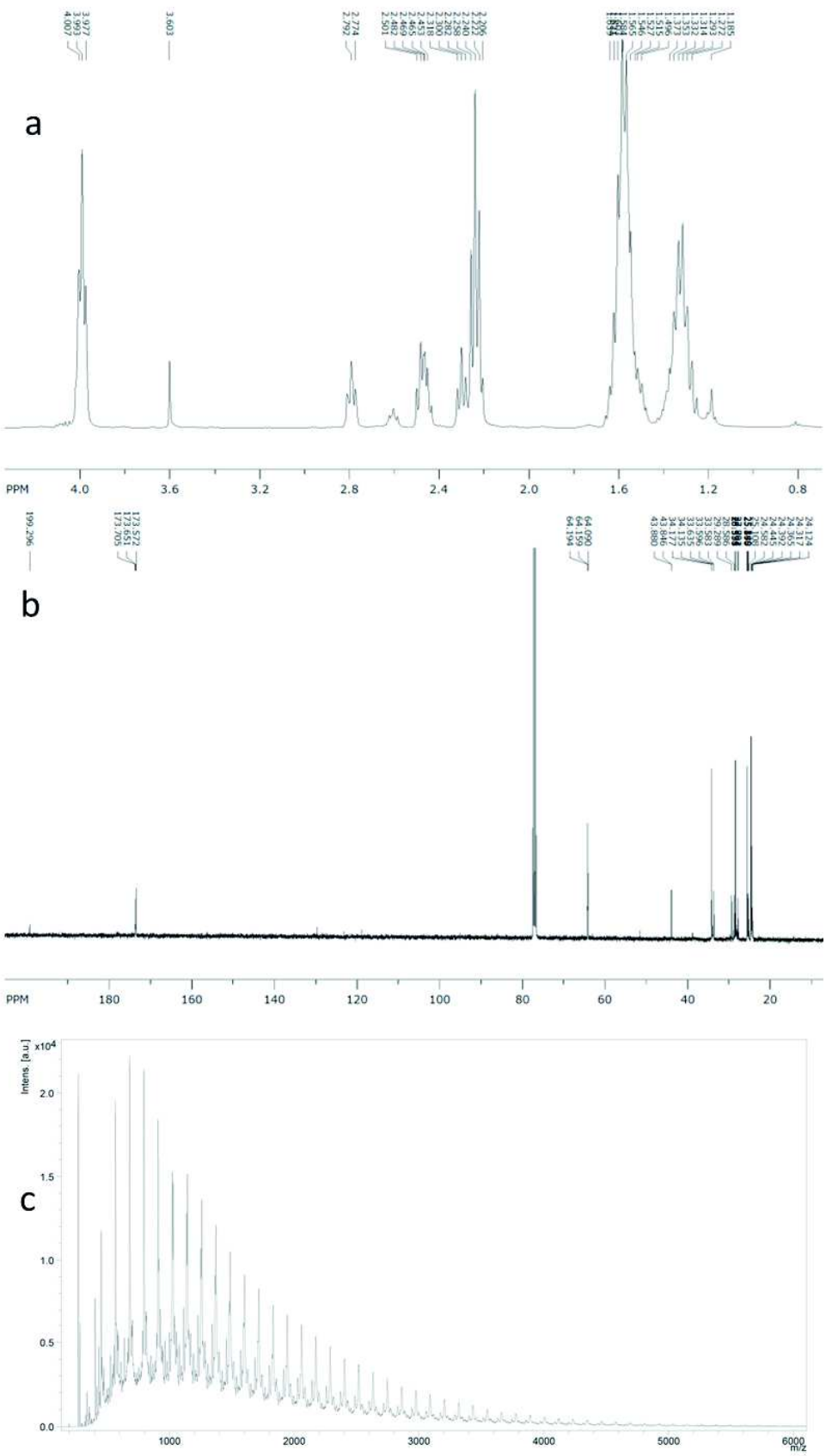

Figure SI IV-7. copolymers N°3 (27% ε-thiocaprolactone content) synthesized by the one step strategy; a- 1H NMR spectrum, b- 13C NMR spectrum, c- MALDI-ToF spectrum

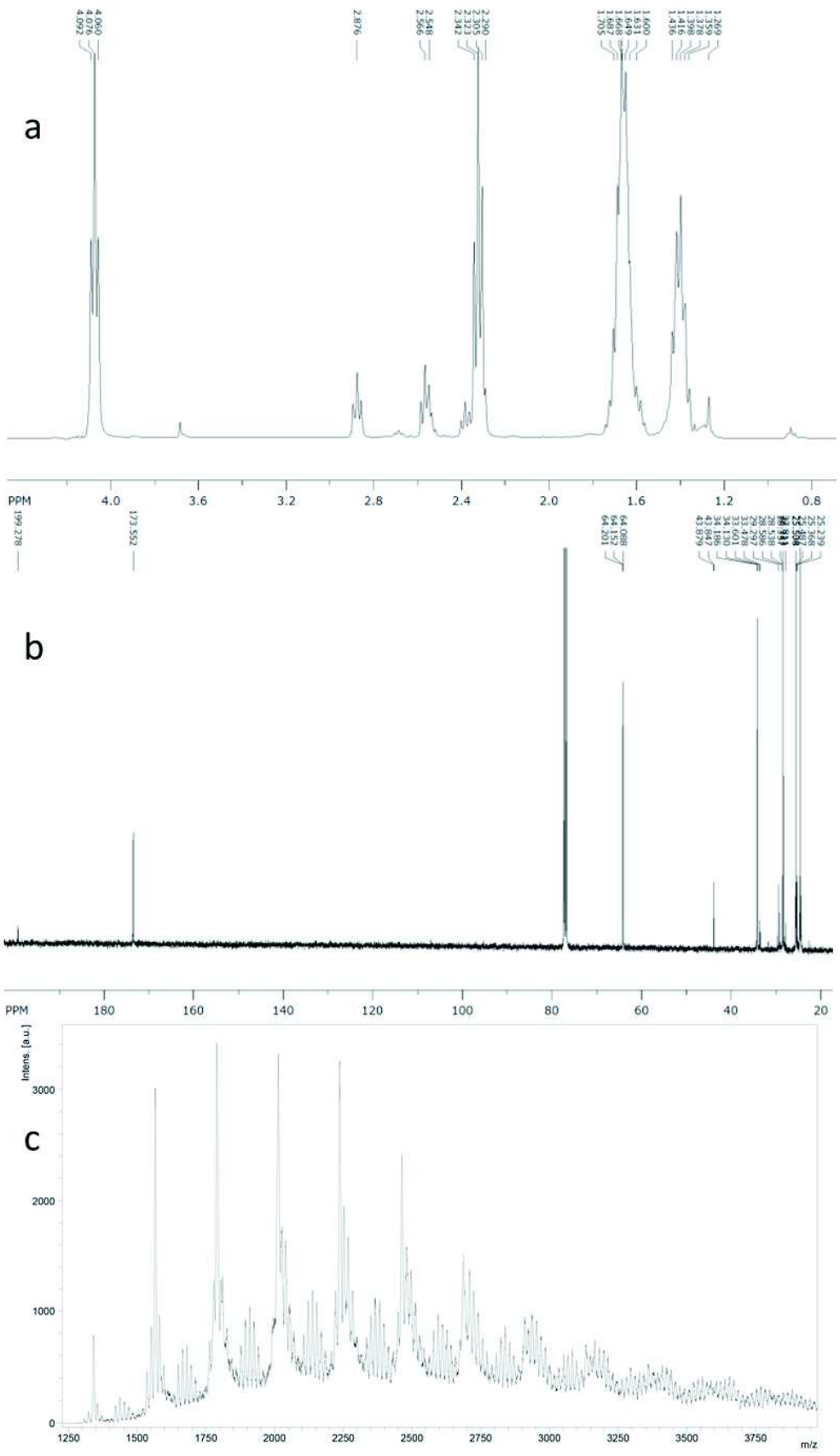

Figure SI IV-8. Copolymers N°6 (16% ε-thiocaprolactone content) synthesized by the two steps strategy; a- 1H NMR spectrum, b- 13C NMR spectrum, c- MALDI-ToF spectrum

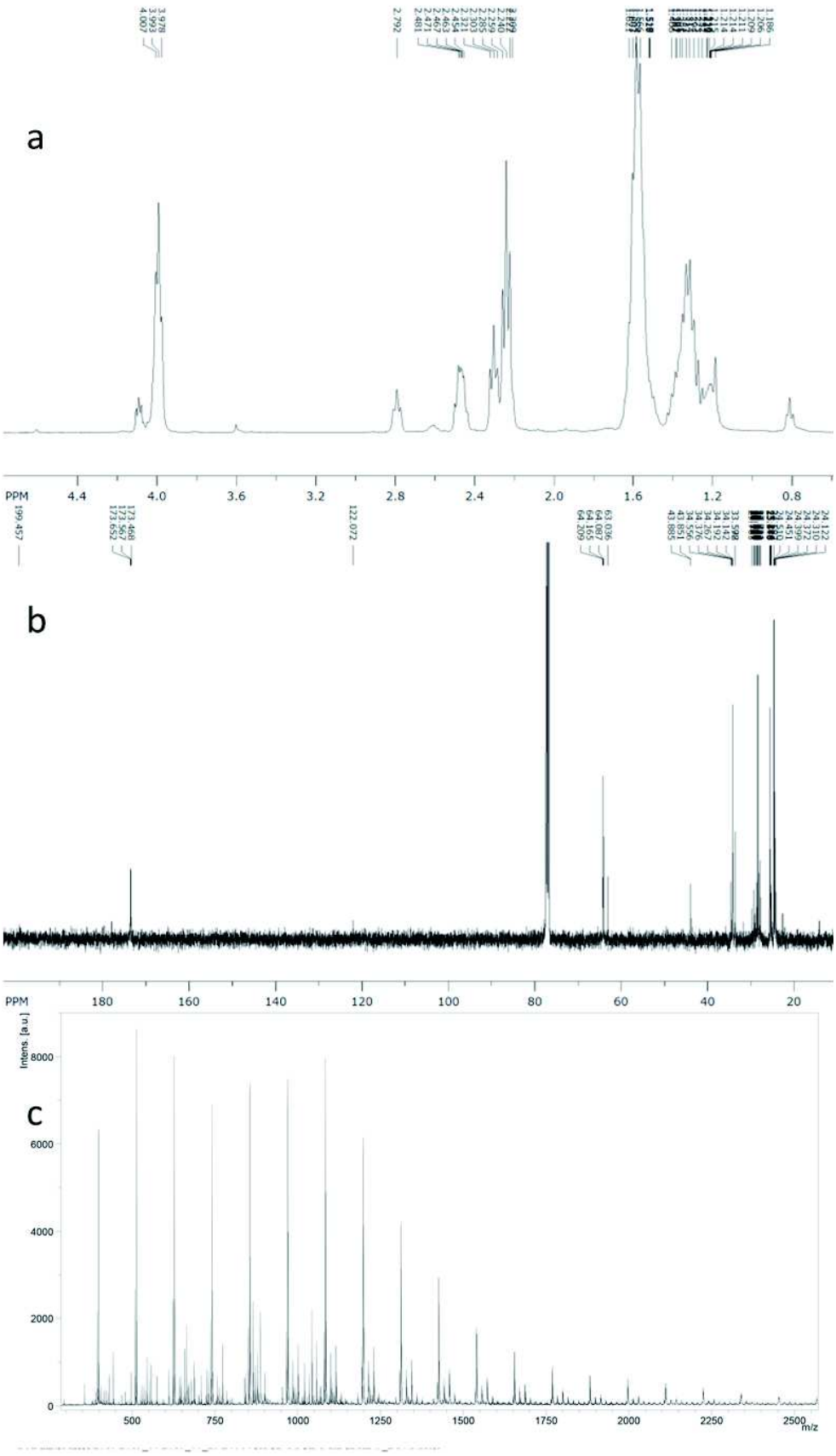

Figure SI IV-9. Copolymers N°7 (19% ε-thiocaprolactone content) synthesized by the two steps strategy; a- 1H NMR spectrum, b- 13C NMR spectrum, c- MALDI-ToF spectrum

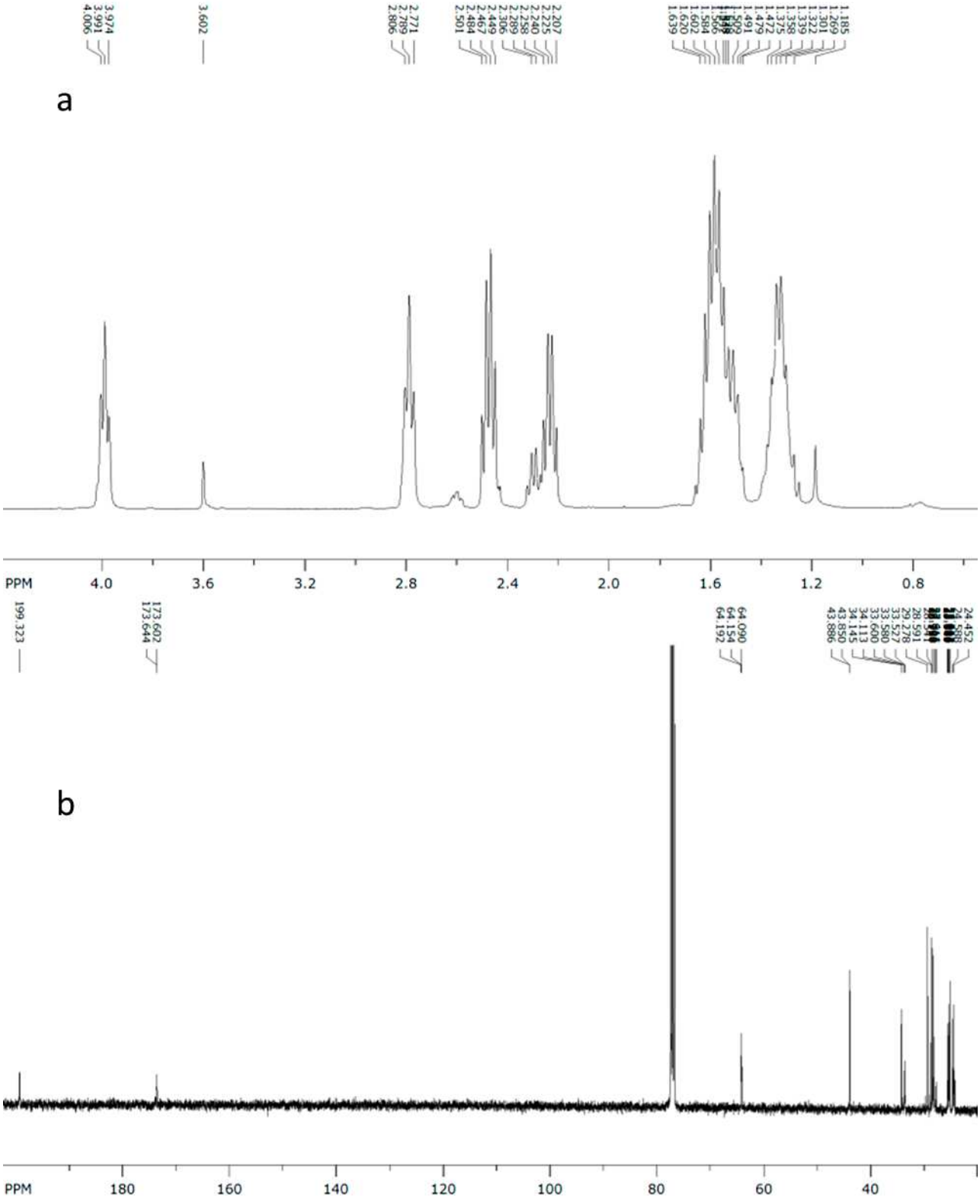

Figure SI IV-10. copolymers N°8 (55% ε-thiocaprolactone content) synthesized by the two steps strategy; a- 1H NMR spectrum, b- 13C NMR spectrum

Conclusions du troisième chapitre

Comme le montrait la littérature, la « proximité chimique » des lactones et des thiolactones fait de ces dernières un substrat relativement adapté à l'eROP. Nous avions ici pour objectifs d'apporter de nouvelles données permettant d'améliorer, dans une certaine mesure, les connaissances et la compréhension de l'eROP sur ces types de monomères.

Des éclairages nouveaux ont été apportés, mais les résultats obtenus mettent en évidence les zones d'ombre et lacunes restants dans la connaissance générale des mécanismes de la polymérisation par ouverture de cycle catalysée par les lipases et plus particulièrement catalysée par la lipase B de *Candida antarctica.*

Ainsi, les résultats des copolymérisations basées sur la stratégie à une étape ont notamment montré que le mécanisme de polymérisation des lactones et des thiolactones n'est sans doute pas aussi simple que celui généralement présenté dans la littérature. En effet, la distribution des pics sur les analyses de spectrométrie de masse ne peut pas être expliquée par ce simple mécanisme d'additions successives d'une unité monomère à la chaîne en croissance ni par les réactions secondaires bien connues d'hydrolyse et de transesterification qui peuvent également être catalysées par l'enzyme.

Ces observations nous ont donc conduits à proposer un mécanisme d'eROP multi-étapes comportant une première étape d'oligomérisation produisant des dimères et trimères (ces dernier étant a priori majoritaires au vu de la structure finale des polymères analysés en spectrométrie de masse), suivie par une étape de croissance ou allongement des chaînes par eROP ou par transesterification. C'est finalement l'occurrence des réactions de transesterification qui rendrait la distribution aléatoire après un temps de réaction significatif.

Par ailleurs, la stratégie de copolymérisation en deux étapes a permis d'atteindre un certain contrôle de l'architecture macromoléculaire avec des copolymères plutôt de type à blocs . Ceci a été confortée par les résultats d'analyse thermique et de spectroscopie de masse qui ont permis d'affirmer la présence de copolymères et non d'un mélange d'homopolymères et mettant en évidence la présence de phases ou séquences distinctes, respectivement plus riches en l'un des deux monomères.

Toutefois, les résultats obtenus doivent être nuancés, non seulement du fait des faibles masses molaires des copolymères obtenus par la synthèse en deux étapes, mais également de par les propriétés thermiques des polymères formés, aussi bien par la première que par la seconde méthode. En effet celles-ci sont très différentes de celles rapportées dans la littérature. Ces écarts pourraient être notamment liés aux faibles masses molaires obtenues.

Les stratégies de polymérisations mises en jeu ici avec l'ε-caprolactone et l'ε-thiocaprolactone, bien que classiques, ouvrent de par leurs résultats prometteurs des perspectives dans la synthèse enzymatique des polymères. En effet, la possibilité de polymériser une grande variété de fonctions chimiques est un atout indispensable au développement de ces méthodes. Cela montre en effet la grande versatilité de ces biocatalyseurs qui étaient ou sont encore souvent considérés, comme extrêmement sélectifs. De plus, la possibilité de contrôler, dans une certaine mesure, la microstructure des polymères formés par eROP est également très intéressante et pourrait donner accès à de nouvelles propriétés macroscopiques pour les polymères issus de ces méthodes de synthèse.

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V. Chapitre 4 – Etude de la variation de l'hétéroatome : le cas de l'azote

Préambule

Cette partie est consacrée à l'étude de l'effet de l'azote, en tant qu'autre hétéroatome, sur la polymérisation par ouverture de cycle catalysée par les enzymes. Dans un premier temps, et dans une approche comparative avec les travaux précédents effectués sur les thiolactones (voir le chapitre 3), nous avons étudié la polymérisation de lactames par eROP.

Toutefois, au vu des résultats présentés ci-après, nous avons également testé d'autres voies de synthèse des polyamides ou poly(ester-co-amide) par catalyse enzymatique, ceci nous a amené vers l'étude d'une copolymérisation d'acides aminé avec des ε-caprolactone.

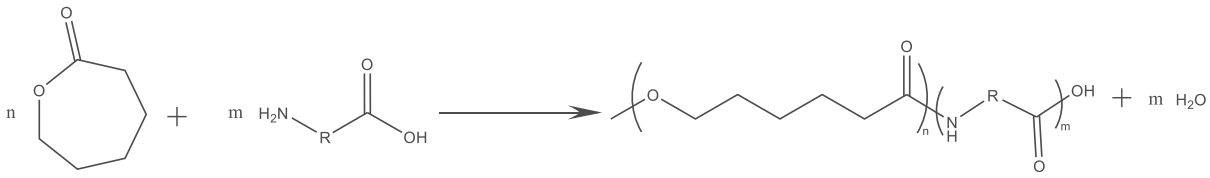

***Figure V-1**. copolymérisation de caprolactone et d'un acide aminé*

Pour rester cohérent avec notre approche verte avec des produits biosourcés, nous avons choisi pour ces expériences des α-acides aminés d'origine naturelle. De plus, afin d'avoir une comparaison la plus complète possible de l'influence des trois hétéroatomes (O, S et N) sur la réactivité en polymérisation enzymatique des monomères correspondant, nous avons choisi de travailler avec des acides aminés soufrés : la méthionine et la cystéine.

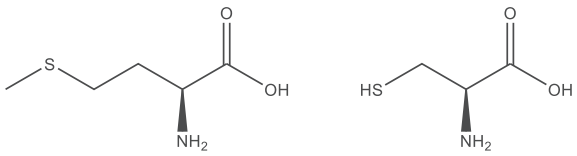

***Figure V-2**. acides aminés soufrés utilisés dans la suite de l'étude : la méthionine et la cystéine*

Ces deux acides aminés ont l'intérêt de présenter deux groupes soufrés différents. La méthionine présente une fonction thioether a priori très peu réactive alors que la cystéine présente un thiol primaire qui est a priori relativement plus réactive. Ce choix de monomères, au-delà de nous permettre de comparer la réactivité des trois hétéroatomes, devrait aussi nous permettre d'obtenir des structures macromoléculaires particulières et contrôlées.

Ainsi, l'utilisation d'acides aminés fonctionnalisés a également l'avantage de pouvoir envisager, dans un second temps, des modifications chimiques simples sur les polymères finaux afin de faire varier et maitriser leurs propriétés physico-chimiques. Ceci peut notamment être réalisé via le greffage de molécules actives sur les chaînes pendantes, la synthèse de polymères en « peigne » par allongement des chaînes latérales, ou encore la synthèse de polymères à blocs, si la réactivité des monomères est suffisamment différente.

Travaux préliminaires

Dans un premier temps, nous avons cherché à réaliser l'ouverture de cycle de lactames équivalents à la caprolactone et au glycolide que sont le caprolactame et l'anhydride de glycine. Nous avons effectué un certain nombre d'essais préliminaires afin de trouver une ou plusieurs enzymes capables de catalyser la ROP des lactames. Les résultats de ces tests ainsi que les conditions expérimentales correspondantes sont résumés dans la Table 1.

Table V-1. étude préliminaire sur les lactames

Monomère	Enzyme	Température (°C)	Système de solvant	Temps (h)	ξ (%)[a]	η (%)[b]	Mn[c] ($g.mol^{-1}$)	Dp
Caprolactame	CALB	70	Toluène	48h	-	-	-	
Caprolactame	Papaïne	70	Toluène	48h	10%	5%	650	5-6
Caprolactame	Papaïne	40	Tampon phosphate pH 7 ; I 0,1	48h	12%	4%	420	3-4
Anhydride de glycine	CALB	70	Toluène	48h	-	-	-	
Anhydride de glycine	Papaïne	70	Toluène	48h	-	-	-	
Anhydride de glycine	Papaïne	40	Tampon phosphate pH 7 ; I 0,1	48h	-	-	-	

[a.] conversions du monomère, déterminé par RMN, [b.] rendement en polymère, [c.] déterminée par SEC

Dans un premier temps nous avons testé l'activité de CALB dans des conditions similaires à celles utilisées pour la polymérisation des lactones ou des thiolactones mais sans parvenir ne serait-ce qu'à ouvrir le cycle lactame. Au vu de ces résultats nous avons donc fait le choix de nous orienter vers d'autres enzymes, produites par Lyven (Groupe Soufflet-France), que sont les protéases. Celles-ci font aussi partie de la famille des hydrolases et ont un site actif et un mode d'action relativement proches de celui des lipases. Leur action naturelle étant l'hydrolyse des liaisons amide des protéines, on peut donc penser qu'elles devraient être capables d'ouvrir les cycles lactames.

Nous avons ainsi effectué quelques tests avec une protéase, courante et peu chère, la papaïne. Il s'agit d'une hydrolase à cystéine qui est également connue pour être capable de former des liaisons amide et d'oligomériser des acides aminés par polycondensation. Pour les mêmes raisons que dans le cas de l'eROP des lactones, nous avons cherché à minimiser la quantité d'eau présente dans le milieu afin de maximiser la longueur de chaîne cinétique.

Mais, là encore, les résultats obtenus sont très limités. Nous ne sommes pas parvenus à ouvrir l'anhydride de glycine (équivalent lactame du glycolide. Dans le cas du caprolactame seuls de très petits oligomères ont pu être obtenus (environ 5 unités) avec une très faible conversion du monomère (autour de 10%).

Enfin, dans une troisième approche nous nous sommes placés dans des conditions plus proches des conditions naturelles de l'enzyme en travaillant à plus basse température dans un tampon aqueux afin de favoriser l'ouverture du lactame. Mais, une fois de plus, seule une petite fraction du caprolactame a réagi pour donner de très petits oligomères (DPn = 3-4) et l'anhydride de glycine n'a pas réagi.

Ces expériences confirment que l'ouverture de cycle enzymatique des lactames n'est pas favorable. En effet, de précédentes études de modélisation ont montré que dans le cas du ε-caprolactame il sera très difficile d'obtenir un degré de polymérisation supérieur à 5. En effet ces modélisations montrent que la première étape de l'eROP, l'acylation de la serine du site actif, est énergétiquement défavorable[1]. Ce degré de polymérisation limité à environ 5 unités est effectivement ce à quoi nous sommes parvenus sans toutefois pouvoir aller plus loin.

Ces essais préliminaires infructueux sur l'eROP des lactames nous ont donc conduits à développer une seconde approche, présentée dans la suite de ce chapitre sous la forme d'un article prochainement soumis à la revue « *Journal of Polymer Science Part A: Polymer Chemistry* », à savoir la copolymérisation d'acides aminés avec la caprolactone. La copolymérisation de la caprolactone avec des acides aminés soufrés a ainsi été étudiée afin de mieux appréhender l'influence des différents hétéroatomes (O, N et S) sur la polymérisation catalysée par les lipases mais également dans le but de générer des architectures macromoléculaires spécifiques et contrôlées.

Caprolactone-amino acids enzymatic copolymerization: a green route towards new functional copolyesters

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Introduction

During the last two decades, there has been a high focus in polymer science for biomedical research, for numerous applications from drug delivery to tissue engineering[2,3]. All these fields require specific physical, chemical and biological properties. To reach such properties several architectures have been developed from polymer chemistry by copolymerization[4], grafting[5] or chemical functionalization[6]. There is a growing need for polymers with easily tunable properties which can combine different macromolecular architectures.

One of the most important properties required for most biomedical uses is the polymers biocompatibility[3], in connection with the non-toxicity of the degradation products and residual monomers[7]. Poly(ε-caprolactone) (PCL) is well-known for its biocompatible polyester[8–13]. PCL has been extensively studied in the lasts decades because of its non-toxicity, biocompatibility and bioresorbability[7]. PCL-based materials has been developed for numerous biomedical applications such as tissue engineering[9], wound dressing[10], single-use medical equipment[11]. For example, Park et al. demonstrated its great potential for bone-repair scaffolds[12]. Several articles report its use for drug releases and delivery systems[12,13].

Nowadays, the ring opening polymerization (ROP) of lactones by metal-based catalysts is the main way to obtain well-controlled polyester chains[14,15]. However these residual catalysts may induce some toxicity (which could be detrimental for e.g., implants or tissue engineering)[16], environmental pollution (in the case of e.g., compostability) and strong increases of the polymer degradation kinetics[17] which could limit the material use. As potential alternatives, some common organocatalysts (like 4-dimethylaminopyridine, triflic

acid or N-heterocyclic carbene)[18] show great potential for lactone ROP, but they also present some toxicity drawbacks.

Enzymatic catalysis currently shows a great potential to substitute toxic metal-based catalysts or organocatalysts to limit the final toxicity and negative environmental impacts, in perfect agreement with a sustainable development and concepts of green chemistry[19]. By enzymatic catalysis, reactions can be performed under mild conditions (low temperature and pressure). Systems with high catalytic activity and very good reaction control of enantio-, chemo-, regio-, and stereo-selectivity can be expected. Owing to these advantages, enzymatic processes could provide precise control of the final polymer architectures, allowing the synthesis of polymers by a clean process, without by-products elaboration, with energy savings[20]. Enzymatic polymerization can thus be regarded as an environment-friendly synthetic process for polymeric materials, providing one of the best examples of "green polymer chemistry". Among the vast choice of enzymes, lipases (E.C. 3.1.1.3) can be found in most organisms from microbial, plant and animal kingdom[21]. They are serine hydrolase that catalyze ester bond cleavage in aqueous medium (physiological action is cleavage of triglyceride) and they are also able to catalyze esters bond formation in organic medium (reverse reaction)[22]. Currently, lipases seem to be the most efficient, at least the most known enzymes for polyester synthesis by enzymatic ring opening polymerization (eROP).

Lactones are one of the most widely studied monomers in eROP catalyzed by lipases[23], especially by lipase B from *Candida antarctica* which is the most efficient enzyme for such reaction. Some studies have shown that large lactones with low ring strain are more compatible with lipases binding site[24] than shorter ones with higher ring strain. Interestingly, chemical ROP shows the inverse reactivity trend with monomer ring strain.

Because of the chemical similarity, other heterocycles based on sulfur, and in a lesser extent on nitrogen, have been studied. There are very few work on thiolactones obtained by eROP[33–38].

Enzymatic synthesis of polyamides[25] by ROP is much more difficult than polyesters synthesis and there are few works on eROP of lactam. These studies show that lactam unit integration is limited[26]. Besides, It is very difficult to obtain long polyamides chains because

of huge stability of large lactam compared to β-lactam[1]. Lipases could catalyze the synthesis of lactam from five to nine membered cycles, and the formation of cyclic amino acids dimers and trimers[27]. Some other strategies have been used to synthesize polyamides or polyesteramides such as polycondensation reaction catalyzed by lipases (e.g., synthesis of low molar mass poly(aspartic acid) [28]) or eROP catalyzed by protease instead of lipases or cutinases which are the most commonly used enzymes[28–31]. Some authors also tried to modify enzymes by mutagenesis, aiming at improving polyamide production, but with mixed results so far[32].

In the present work, we report assays of different synthetic routes to elaborate new copolymers based on ε-caprolactone and two selected amino acids. Cysteine and methionine have been selected since their respective side groups presents adequate chemical functions: (i) a thiol for cysteine, which allows subsequent branching, grafting or cross-linking, and (ii) a thioether for methionine that could allow a control of the polymer thermal resistance, for example by sulfonation of the thioether[39]. A protection-unprotection approach of the chemical groups has been used and then two different strategies were tested: (i) preliminary tests based on direct polymerization, using methionine and cysteine amino-acids, and (ii) an indirect approach, involving protected cysteine. The macromolecular architectures and the mechanisms to obtain the corresponding (co)polymers them have been analyzed trough different techniques. The main goal of this study is to compare and draw conclusions on the relative reactivity of acid, ester, amine and thiol functions by enzymatic catalysis.

Materiel & methods

Materials:

ε-caprolactone (CL) was purchased from Aldrich and distilled over CaH_2 under reduced pressure before each use. Methionine (Met) and cysteine (Cys) amino acids as well as Novozym®435 (N435), the acrylic resin-immobilized form of *Candida antarctica* lipase B (CALB), were purchased from Aldrich and used after freeze drying. Anhydrous toluene was freshly distilled over sodium under nitrogen atmosphere prior each use. Hexanol was freshly distilled on molecular sieve under vacuum prior use. Other chemicals (N-Boc cysteine, N-Boc Methionine, 4Å molecular sieve, *p*-toluenesulfonic acid, $NaHCO_3$), and other solvents

(methanol, diethyl ether, ethanol, chloroform, dichloromethane), were purchased from Aldrich and used without further purification or drying.

N-Boc-cysteine hexyl ester (N-Boc Cys HE) synthesis:

Typically, 1g of N-Boc cysteine (4.5 mmol) was added in a round bottom flask with 20 equivalents of freshly distilled hexanol (11.4 mL; 90 mmol) under argon atmosphere. Then 2 mol% of *p*-toluenesulfonic acid were added to reaction the flask and system was capped with a distillation bridge. Then the system was heated to 140 °C for 3 h, few droplets of water were collected by the distillation bridge. After cooling to RT, reaction medium was neutralized by $NaHCO_3$. After filtration the organic phase was taken by diethyl ether and washed several times with water. Then, residual alcohol and solvent were distilled to produce about 1.05 g (76% of yield) of a slightly yellow oil. ^1H NMR ($CDCl_3$; 400 MHz): δ = 0.87-0.90 ppm (t, 3H, H_3C-CH_2-); δ =1.25-1.39 ppm (m, 6H, CH_3-C_3H_6-CH_2CH_2CO-); δ =1.45 ppm (s, 9H, tertiobutyl); δ = 1.53-1.70 ppm (m, 2H, C_4H_6-CH_2CH_2O-); δ = 2.91-3.12 ppm (m, 2H HS-CH_2CH); δ = 4.15-4.27 ppm(m, 2H, CH_2-CH_2O); δ = 4.35 ppm(s, 1H, NH); δ = 4.48-4.62 ppm(m, 1H, HS-CH_2-CH-).

Enzymatic polymerization setup:

All reactions were carried out in dry toluene (2 mL), at 70 °C. For that, 3.95 mmol of CL (0.9017 g) and the adequate quantity of amino acid in molar equivalent (from 40 µmol to 0.395 mmol: e.g. for cysteine from 4.8 mg to 47.8 mg for 1% to 10 mol%, respectively) and 4Å molecular sieve (0.05 g) were introduced into a previously dried Schlenck tube under an inert dry argon atmosphere. The tube was immediately capped with a rubber septum and then immersed in a heated oil bath at 70 °C. Toluene was transferred with a syringe through the rubber septum cap. A predetermined amount of N435 catalyst (50 mg) was quickly introduced in the tube under an inert dry argon atmosphere. The tube was immediately capped with a rubber septum. The enzyme addition marked the beginning (t0) of the polymerization. Reactions were terminated by dissolving the reaction mixture into chloroform and removing the catalyst by filtration. Part of the solvent in the filtrate was then stripped by rotary evaporation at 35 °C. The polymer in the resulting concentrated solution was precipitated in cold methanol (in a dry ice-ethanol bath at approximately -70°C) to

ensure also the recovery of the shortest chains. The precipitated polymer was recovered by filtration and dried overnight at 30 °C under vacuum. For the polymerization involving N-boc cysteine hexyl ester, similar procedure than for eROP of unprotected amino acids and CL was performed.

Characterization techniques:

NMR analyses were performed on a Bruker Ascend™ 400 spectrometer operating at 400.13 MHz and 100.62 MHz for ^{1}H and ^{13}C NMR, respectively. Spectra were obtained by performing at least 64 scans for ^{1}H, 1024 scans for ^{13}C, 8 scans for COSY and 8 scans for HSQC analyses. 1D and 2D NMR spectra were exploited with SpinWork 4.1 software. All the samples were prepared in deuterated chloroform with typically 8 mg for ^{1}H and 15 mg for ^{13}C and 2D NMR analyses. The determination of N-boc cysteine hexyl ester content in the copolymer was achieved using the integration of the singlet at 1.45 ppm, corresponding to the 9 protons of Boc tertiobutyl group, compared to the sum of the integrals of the two triplets at 3.65 and 4.06 ppm corresponding to the ROCH_2R in poly(ε-caprolactone) chain-end and main chain units, respectively.

$$N-boc\ cysteine\ hexyl\ ester\ (\%) = \left(\frac{I_{Boc}/9}{(I_{Boc}/9)+(I_{PCL}+I_{PCL\ endchain})/2}\right)\times 100 \quad (1)$$

Size exclusion chromatography (SEC) measurements were performed in chloroform (HPLC grade) in a Shimadzu liquid chromatograph equipped with a LC-10AD isocratic pump, a DGU-14A degasser, a SIL-10AD automated injector, a CTO-10A thermostated oven with a 5 µ PLGel Guard column, two PL-gel 5 µ MIXED-C and a 5 µ 100 Å 300mm-columns, and three online detectors: a Shimadzu RID-10A refractive index detector, a Wyatt Technologies MiniDAWN 3-angle-light scattering detector and a Shimadzu SPD-M10A diode array (UV) detector. Samples were dissolved in chloroform (concentration 4 mg.mL^{-1}) and filtered through a 0.45 µm PTFE membrane. For all analyses the injection volume was 100 µL, the flow rate was 0.8mL.min^{-1} and the oven temperature was set at 25 °C. Molar masses and dispersity were calculated from a calibration with polystyrene or polymethylmethacrylate standards in UV or RI detection. Refractive index increment values (dn/dc) were measured by injecting a known concentration of polymer and assuming 100% mass recovery from the system

MALDI-ToF analysis starts with the samples preparation. Matrix solutions were freshly prepared with Super DHB (9:1 mixture of 2,5-Dihydroxybenzoic acid and 2-hydroxy-5-methoxybenzoic acid, from Sigma Aldrich) which was dissolved till saturation in a $H_2O/CH_3CN/HCOOH$ (50/50/1%) solution. Typically, a 1:1 mixture of the sample solution in CH_2Cl_2 was mixed with the matrix solution and 1 µL of the resulting mixture was deposited on the stainless steel plate. Mass spectra were acquired on a time-of-flight mass spectrometer (MALDI-ToF-ToF Autoflex II ToF-ToF, Bruker Daltonics, Bremen, Germany) equipped with a nitrogen laser (λ = 337 nm). An external multi-point calibration was carried out before each measurement using the singly charged peaks of a standard peptide mixture (0.4 µM, in water acidified with 1% HCOOH). Scan accumulation and data processing were performed with FlexAnalysis 3.0 software.

The thermal stability and degradation of the samples was investigated by a thermal gravimetric analyzer (TGA) coupled with a FTIR for evolved gas analysis. TGA measurements were conducted under dry air (at a flow rate of 75 $mL.min^{-1}$) using a Hi-Res TGA Q5000 apparatus from TA Instruments. The samples (5-9 mg placed in a platinum pan) were heated up to 450 °C at 5 $°C.min^{-1}$. FTIR spectra were recorded on a Nicolet 380 (Thermo Electron Corporation) by performing 16 scans with 4 cm^{-1} resolution.

DSC measurement were performed on a TA Q200 DSC in sealed aluminum pan, typically on about 1 mg of purified sample, in a heat-cool-heat cycle at 10 and 5 $°C.min^{-1}$ for heating and cooling, respectively.

Results & discussions

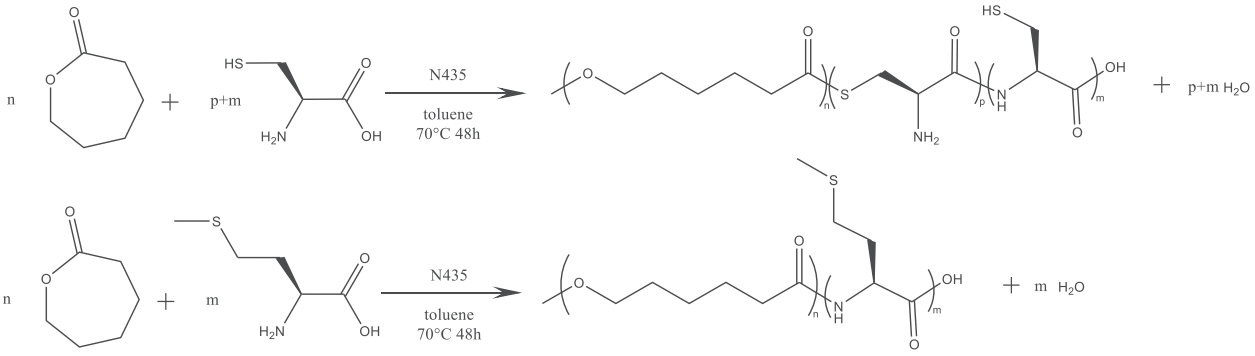


Figure V-3. *Illustration of the direct and preliminary approaches based on unmodified amino acids with CL.*

In a preliminary approach, we tried the enzymatic synthesis by a direct way, as shown in Figure 3. Main results are summarized in Table 2, where, first line correspond to blank test, without amino acid, the second to the fifth lines correspond to CL copolymerization with cysteine and the last four to CL copolymerization with methionine in order to compare different amino-acids based on sulphur and the effect of the feed content.

Table V-2. *direct amino acids–caprolactone copolymerization results*

Amino acid	Amino acid feed content (mol %)	Mn[a]	Đ[a]	Tm	Tc	χ	Td °C [b]
	0	15 000	1.7	56	34	59%	400
Cys	1	11 300	2.1	50.9	36.0	47%	401
Cys	2	11 200	2.1	51.3	32.1	47%	399
Cys	5	11 500	2.2	51.7	32.3	43%	400
Cys	10	10 500	2.1	52.2	35.2	43%	399
Met	1	11 200	2.2	51.4	36.4	41%	395
Met	2	11 000	2.2	49.8	35.4	43%	398
Met	5	10 800	2.2	51.7	35.5	41%	394
Met	10	10 500	2.1	51.2	35.2	51%	396

[a] determined by SEC; [b] degradation temperature at maximum degradation rate; [c] degradation temperature at 20% weight loss

Table 2 clearly shows that addition of small amounts of amino acids in the reaction medium does not lead to significant differences on the resulting polymers. Molar masses are quite constant, ranging from 10 500 to 11 500 with cysteine and from 10 500 to 11 200 with methionine (that is slightly lower that blank test that show a molar mass around 15 000 $g.mol^{-1}$, that could be due to the initiation by amino acids or, more probably to water added with amino acids that are very hygroscopic compound). One can also notice the relatively high dispersity, around 2, which could mean that there are two different initiation steps or competitive transesterification reactions. As reported in a previous work[40], N435 is an efficient transesterification catalyst. This high dispersity is also linked to a long reaction time (48 hours) compared to the more conventional 24 hours for eROP based only on CL units[41]. Such a high dispersity could also been explained by an initiation either by the amine of methionine or cysteine or by the thiol of cysteine, both being in competition with the conventional water initiation.

From the TGA results, one can observe a non-negligible weight loss before the main degradation peak at around 400°C mainly due to water (below 150°C) and residual CL monomer (below 250°C) losses. These molecules have been chemically identified by IR analysis of the evolved gas from the degradation (Supporting Information (SI), Figure SI1).

One can also notice from the DSC results that thermal properties seem not to be affected by the addition of amino acid during the synthesis and these values are very close to those expected for PCL homopolymer. For example, melting temperature ranges between 49 and 52 °C which is comparable, or slightly below, to the melting temperature of PCL of such molar mass. In the same way, degradation temperatures (around 400°C) are very close to the one of neat PCL. These results tend to demonstrate that there are no, or very few, insertion of amino acids in the PCL polymer chain.

This has been confirmed by the ^{1}H and ^{13}C NMR analyses displaying only the characteristic peaks of PCL chains (Spectra in SI, Figure SI2), highlighting that mainly PCL homopolymers have been obtained. The presence of residual CL has also been confirmed by ^{1}H NMR spectroscopy (SI, Figure SI2), with an estimated content around 5 to 10% after the polymer precipitation recovery step. Such presence is somewhat expected since our precipitation protocol in cold methanol (-78 °C) is also able to precipitate CL.

One can notice that, initial amino-acids do not appears on products analysis. There are probably precipitated during the termination step (with chloroform addition) and eliminated by filtration with the enzyme.

The results of these preliminary tests show that the insertion of significant amino-acid units in the polymer chain has not been clearly established, then the corresponding products were not considered for further characterization. It is assumed that this limited insertion of amino acids and their lack of reactivity are mainly due to their poor solubility in CL and toluene but that could also be due to a weak affinity with the lipase binding site. In order to alter the low solubility of amino acids in toluene, N-boc cysteine hexyl ester (Figure 4) in a protected-unprotected approach has been used. Indeed, tertiobutyl and hexyl group drastically increase the solubility of amino acids in organic solvent. Besides, the hexyl group also increases the affinity of this substrate with the enzyme. To improve the amino acids incorporation in the chain, to increase the solubility of the amino acids in the reaction medium, N-Boc protected derivative of these amino acids has been used. The protecting group presents several advantages such as an increase of the solubility of amino acids in toluene. Besides, compared to the native carboxylic acid, hexyl ester could make the monomer closer to the lipase (i.e. fatty ester) to increase the monomer affinity with enzyme binding site and then to increase the reactivity. However, direct polymerization strategy did not show any reactivity of the thioether function of methionine. Since N-protected methionine would have only one reactive chemical group for the polymerization, then only cysteine derivatives have been used for the corresponding approach.

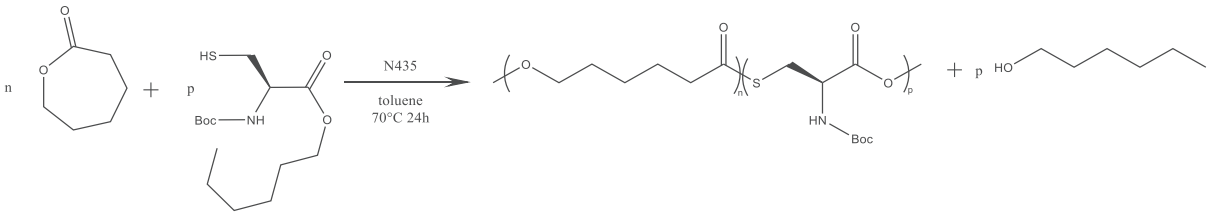


Figure V-4. *Copolymerization strategy based on N-Boc protected cysteine hexyl ester*

Results of the strategy based on the modified cysteine are summarized in Table 3. At first we noticed a significant decrease of the molar masses with the increase in amino acid feed content. Such decrease is not surprising since there are four different potential initiating species. Two of them are initially present in the reaction medium (residual water and N-boc Cys HE) and the others are produced during the polymerization reaction (hexanol from transesterification of N-Boc Cys HE, water produced by reaction on thiol function of N-boc Cys HE). Among these four potential initiators, three of them thus show an increase with the increment of the amino acid feed content. One can also noticed that for the 1% amino acid content we obtain a final molar mass higher than in preliminary step and close to the blank test (see Table 2), that is probably due to the lower hygroscopicity of protected amino acids compared to native one.

***Table V-3**. Main results from the copolymerization based on protected cysteine with CL*

amino acids	Amino acid feed content (mol %)	Mn ($g.mol^{-1}$)[a]	Đ	Tm (°C)	χ	Td °C [b]	first mass loss [c]	Amino acid content (mol%)[d]
N-Boc Cys HE	1	14300	2.3	55.8	59%	405	1.1%	0.7%
N-Boc Cys HE	2	11400	2.5	53.9	42%	404	1.4%	0.8%
N-Boc Cys HE	5	8000	1.6	51.1	51%	402	2.0%	1.6%
N-Boc Cys HE	10	6500	1.6	51.9	39%	405	3.3%	1.9%

[a] determined by SEC in PS equivalent, [b] maximum degradation rate, [c] mass loss at 250°C, [d] determine by NMR

According these results, one can notice that there are on average only 1 to 1.3 amino acid units per chain. This means that the amino acid reacts mainly as initiator or as chain ending. This low incorporated amino acid content is also confirmed by TGA analyses with the mass loss below 250 °C that corresponds to the loss of the Boc protecting group, the residual water and the unreacted monomers as shown by the TFIR coupling.

The ^{13}C NMR characterization shows interesting pattern (Figure 5). Most of the characteristic peaks of protected amino acid could be easily identified except for the three

peaks corresponding to the carbonyl of Boc protecting group, to the quaternary carbon of the tertiobutyl group and to the carbonyl of hexyl ester function. The absence of these peaks is not surprising because of the usually weak response of sp^4 carbons in ^{13}C NMR and the small amount of amino acid in the copolymers (with a maximum of 1.9 mol%, Table 3). Interestingly, one could also notice that the signals of hexyl ester are always observed in the polymers which could mean that at least part of N-Boc Cys HE did not react on its hexyl ester function or that hexanol released by transesterification of N-Boc Cys HE initiates an eROP side reaction. This second possibility is more probable due to the well-known ability of N435 to catalyze transesterification reactions.

One can also notice on the ^{13}C NMR spectrum, that there are four smalls peaks between 30 and 34 ppm (assignment on Figure 5), e' corresponding to acid chain-end, e" corresponding to thioesters linkage and g' corresponding to residual thiol function.

These results seem to indicate that there are several distinct populations of polymer chains due to some side reactions, such as hexanol initiation, but also due to the multiple reactive sites on N-Boc-Cys HE monomer.

^1H NMR spectra of the products obtained from CL copolymerization with N-Boc Cys HE (Figure 6 and also from SI, Figure SI3, SI4 and SI5, respectively, for 10%, 1%, 2% and 5% amino acid) show significantly different patterns than those observed in the first polymerization strategy. All characteristic peaks of N-Boc Cys HE with the presence of hexyl ester signals that correspond to the chain population initiated by a thiol function or the population initiated by hexanol resulting from the transesterification of cysteine hexyl ester can be clearly identified on the ^1H NMR spectrum (Figure 6). However, these analyses did not allow us to determine which reaction is more likely and to conclude on this specific point.

To characterize more precisely those copolymers, 2D NMR experiments were performed. Despite some absent (or too low intensity) signals on the ^{13}C NMR, the proposed peaks assignment has been confirmed by HSQC (^1H, ^{13}C), 2 dimensional NMR experiment which directly shows the bonded protons and carbons. Figure 7 shows the confirmation of our

assignment for the low intensity peaks in ^{13}C NMR for carbons g and h which make coupling spots at respectively 65 and 26 ppm.

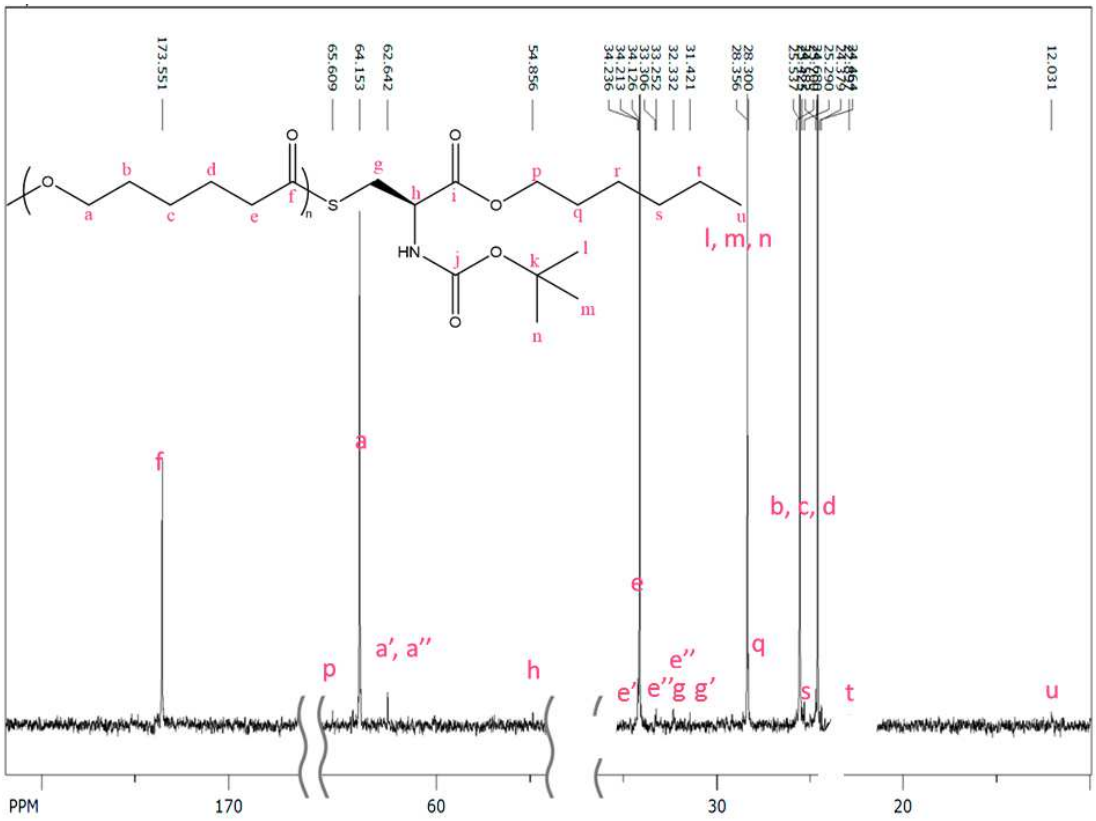


Figure V-5. *^{13}C NMR of copolymerization product between ε-CL and 10 mol% of N-Boc Cysteine hexyl ester in the feed, prime corresponding to an alcohol chain-end, double prime to an acid chain-end.*

Figure V-6. *^{1}H NMR of copolymerization product between ε-CL and 10 mol% of N-Boc Cysteine hexyl ester in the feed, prime corresponds to an alcohol chain-end, double prime to an acid chain-end.*

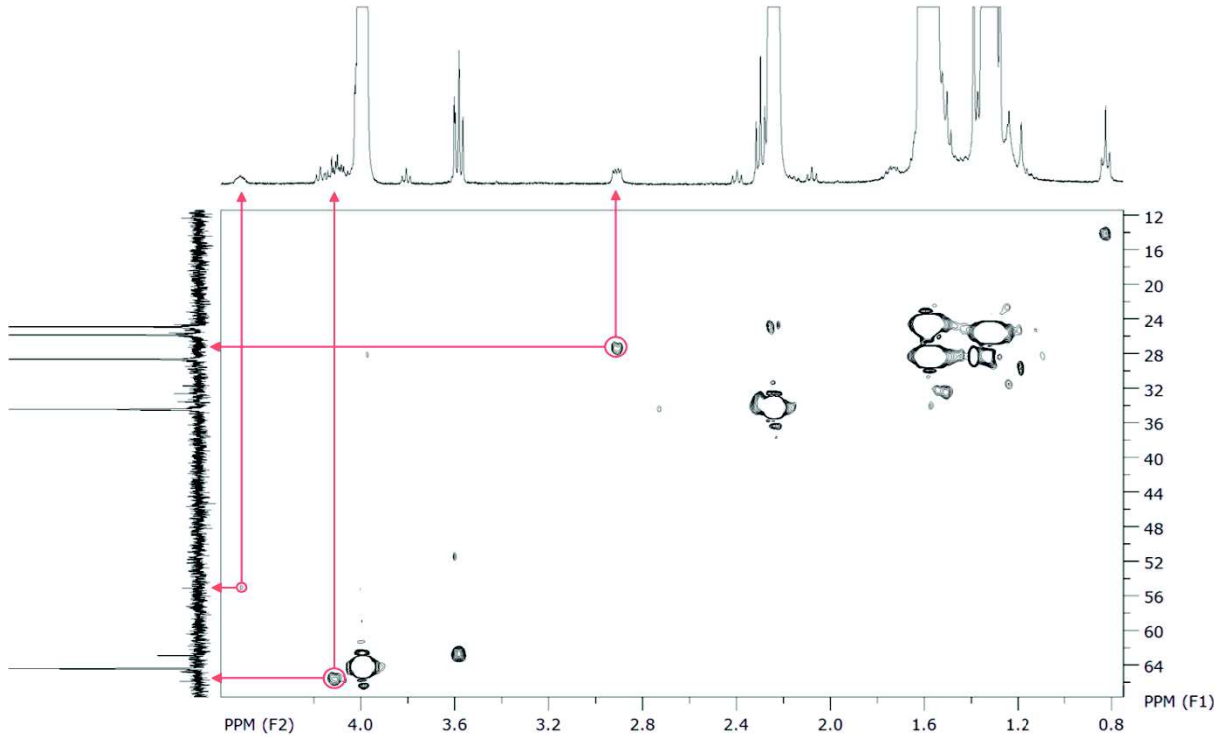
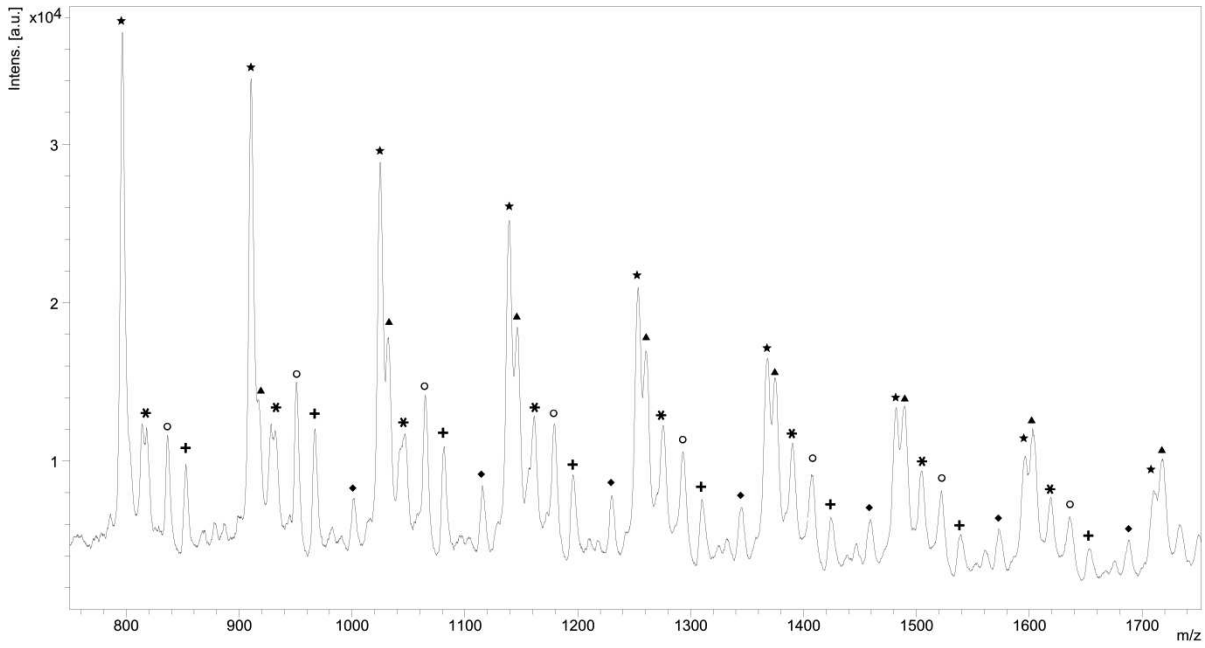
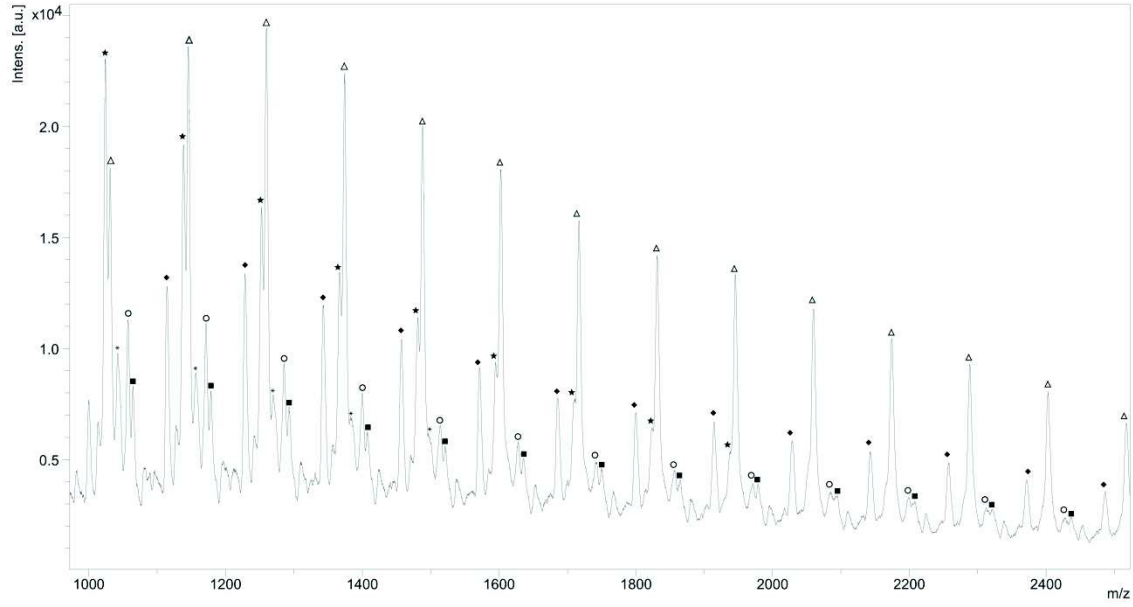


Figure V-7*. HSQC proton-carbon NMR of copolymerization product between ε-CL and 10 mol% of N-Boc Cysteine hexyl ester in the feed.*

The NMR spectroscopy highlighted the multiple polymer chain populations but despite the deep analysis of the spectra, the full determination on their different chemical structures stays partially unelucidated. Then, to clarify such complex polymer chains populations and structures, Maldi-ToF mass spectrometry of the copolymers of CL with N-Boc-Cys HE were performed. Two examples of the resulting mass spectra are shown in Figures 8-a and 8-b corresponding respectively to the copolymers with 2 and 10% of N-Boc cysteine hexyl ester in the feed (Maldi-ToF spectrum of 1% of N-Boc cysteine hexyl ester in the feed is available in SI, figure SI3b).



(a)



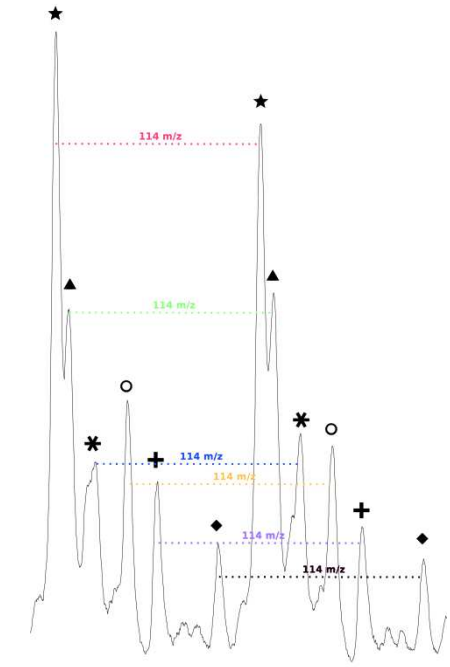
(b)

Figure V-8. MALDI-ToF MS spectrum of copolymer of CL with (a) 2 and (b) 10 mol% of N-Boc cysteine hexyl ester in the feed

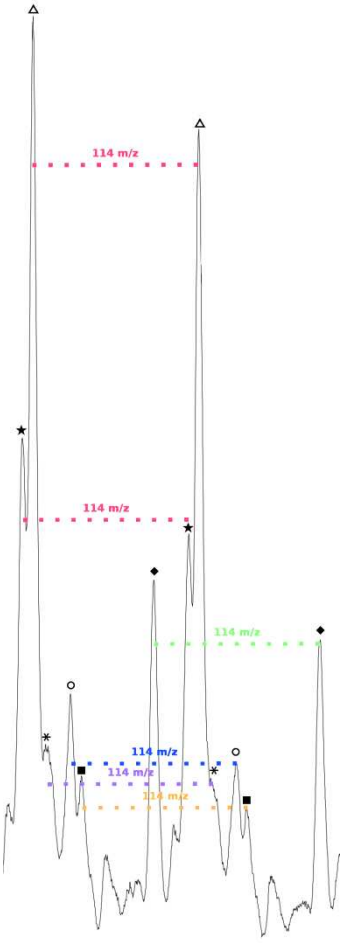
One can see on Figure 8-a that there are at least 6 different distributions of polymer chains. These analyses also show that the m/z value of each peak logically verifies Equation 2.

$$m/z = M_{Na} - M_H + nM_{Cl} + M_{chain-ends} \quad (2)$$

We can also notice on Figures 9-a and 9-b, that for all distributions the interval between two successive peaks of a same distribution is 114 m/z, which exactly corresponds to one CL unit. This result validates our previous hypothesis made from the amino acid content and confirms that there is more or less one amino acid by polymer chain (Table 3).



(a)

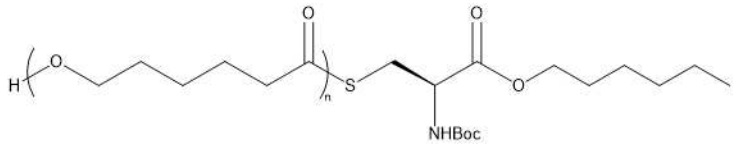
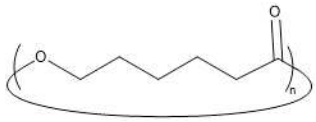
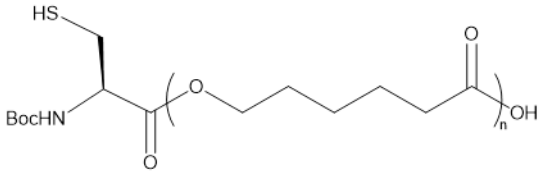
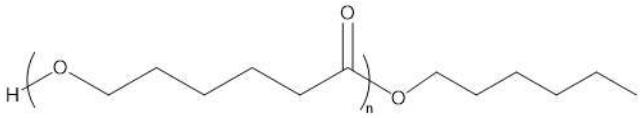
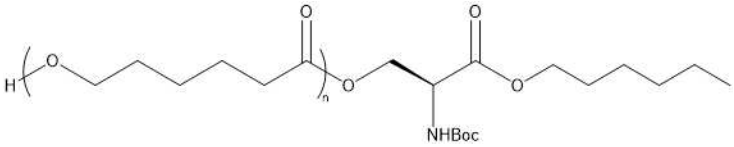
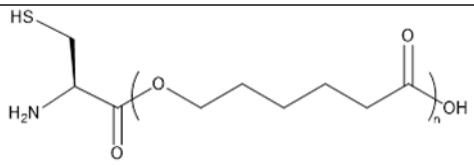


(b)

Figure V-9. interval between the peaks of two successive mass distribution for the copolymerization of CL with (a) 2 and (b) 10 mol% of N-Boc cysteine hexyl ester in the feed

A detailed analysis of these mass spectroscopy spectra allows to identify the different types of chain-ends and/or polymer structure corresponding to each distribution. The main results for polymers obtained with 2 and 10 mol% of N-Boc-cys HE in the feed are summarized in Table 4.

Table V-4. *different structures for the products of caprolactone copolymerization with N-Boc cysteine hexyl ester in the feed*

symbol	structure	supposed eROP mechanism
★		initiated by thiol of N-Boc-Cys HE
○		terminated by chain cyclization
Δ		initiated by water and ending by transesterification of N-Boc-Cysteine hexyl ester
*		initiated by hexanol
+		Initiated by residual water
♦		Initiated by N-Boc-Cys-OH resulting from sulfur substitution by oxygen in the binding site during the deacylation step.
■		initiated by water and ending by transesterification of Cysteine hexyl ester

As expected the main distribution is initiated by the thiol function of cysteine, this initiation seems to be more important than the one from residual water. This could be easily explained by the very low water content since we minimize it for each product and at each step. We also noticed chains termination by an amino acid due to transesterification reaction involving the hexyl ester. Interestingly, this termination seems to become more important for longer polymer chains. That could indicate that after a first oligomerization/polymerization step, the chain growth is then mainly made by transesterification and chain coupling. As supposed from the NMR results, there is a chain distribution initiated by hexanol resulting from transesterification of the hexyl ester amino

acid (Figure 4). From the MALDI-TOF spectra, one can also notice the presence of cyclic PCL which have already been reported in the literature[42]. Surprisingly, there is another distribution with a rather unexpected chain-end based on N-Boc-Serine hexyl ester (N-Boc-Ser-HE). This likely means that there is a substitution of the sulfur atom of the amino acid by an oxygen (from the serine residue of the active site) during the deacylation step of the reaction (Figure 10). However, from the low peak intensity in the MS spectra, it seems that such substitution is of very low occurrence and could explain why such products were not identified in NMR spectroscopy (also because their characteristic peaks could be masked by other signals).

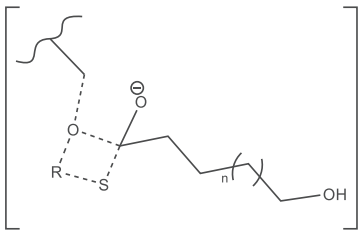


Figure V-8. *possible intermediate species in the enzymatic polymerization deacylation step that could lead to either a thioester- or an ester-initiated chain.*

Interestingly, for higher amino acid contents in the feed, the Maldi-ToF spectra are modified. Figure 8-b, shows that 6 main distributions are also observed in this case but with two main differences in the distinct structures (Table 4). One can first notice the absence of chains initiated by water molecules. This could be explained by the higher amount of amino acid which makes water molecules negligible compared to the others available initiators such as the thiol function of cysteine and hexanol released by transesterification of N-Boc-Cys HE. As a second difference, one can also notice a surprising chain population that seems to be terminated by a cysteine. This distribution could result from a termination reaction by transesterification with unprotected cysteine hexyl ester. The latter would originate from a native amino acid that would have been unprotected during the synthesis of N-Boc-Cys HE. Indeed, amino acid esterification reaction is performed in acidic medium and this could

weaken, and possibly remove, the N-Boc protecting group. However this chain distribution seems to be the least important.

One can also notice from the peaks intensities that the relative amounts of the various distributions are also different for polymers synthesized with larger quantity of amino acid in the feed. For instance, for the low amino acid content, the main distribution seems to be the chains initiated by the cysteine thiol whereas for large amount of amino acid, the chain terminated by transesterification of the hexyl ester seems to be major one. It was also noticed that for longer chains, the sulfur substitution seems to be more important while this had not been observed for low amino acid feed content.

As seen from Figure 10, and as already observed for the low amino acid content, for all distributions the interval between two successive peaks of a same distribution is 114 m/z, which corresponds exactly to one CL unit. This result confirms that, even if we increase the amino acid content in the feed, only more or less one amino acid unit are incorporated in the polymer chain.

Conclusion and perspectives

The present work presents the enzymatic polymerization of ε-caprolactone with immobilized lipase B (Novozym®435) in presence of unmodified or modified amino-acids. Different strategies were tested, a preliminary one with a direct polymerization, and an indirect one based on amino-acid protected functional groups.

The preliminary strategy did not show any significant reactivity of the amino acids, and significant difference between the amino-acids (cysteine and methionine). Despite the fact that lipases in their natural environment are catalysts that work well at aqueous-organic interfaces and which could then be efficient for syntheses in heterogeneous systems, the

results from our first polymerization strategy clearly showed that the most important parameter in such reaction is the miscibility of each component. Indeed, it is assumed that this lack of reactivity could be due to a poor solubility of the amino acids in CL and toluene. Nevertheless, this lack of reactivity could also be due to a low affinity of the native amino acids with the lipase binding site (due to many apolar area or steric hindrance on amino acid) which is designed for fatty acids and fatty esters substrates.

Form these preliminary tests, a main strategy was established based on the of N-Boc and/or hexyl ester protected amino acid, to enhance its solubility and improve its affinity with lipases binding site. This strategy produced numerous populations of polymer chains that are mainly PCL homopolymer chains with distinct initiators and/or chain-ends. At first, PCL initiated by N-boc-cysteine hexyl ester and N-boc-serine hexyl ester were identified as well as PCL terminated with cysteine and N-boc cysteine . In addition, other distribution due to side reactions were also observed such as PCL chains initiated by residual water or by hexanol released in the medium together with cyclic PCL chains. Each of these polymers distribution brings some mechanistic information on the copolymerization that could be useful for the enzymatic synthesis of controlled macromolecular architectures. First of all, the significant monomer conversion observed for the protected amino acids (compared to the lack of reactivity for their unprotected counterparts) confirms that the solubility of these monomers and their affinity with the lipase binding site are key parameters for their enzymatic (co)polymerization. Besides, the occurrence of a distribution initiated by N-Boc serine indicates that there is a potential substitution of the sulfur heteroatom of the initiator by an oxygen from the serine residue of the enzyme active site as a result of the tetrahedral intermediate formed during the ring opening step. Finally, it is possible to take advantages of some side reactions like hexanol initiated and water initiated eROP for example by using a fatty diol like 1,12-dodecanediol that could lead to longer chains by promoting chain coupling.

The different studied strategies lead us to several hypotheses on the mechanism of this enzymatic polymerization. The various in-depth characterizations that have been performed highlight some key parameters, like solubility, steric hindrance of the initiator and monomers structure, that once controlled could pave the way for the synthesis of various

new fully biobased and biocompatible copolymers with potentially tunable macromolecular architectures and functionalization.

The polymerization strategy based on modified amino-acid opens perspectives for subsequent functionalization of the polymer chain by simply removing the N-Boc protecting group (e.g by using trifluoroacetic acid as previously described)[43], that will make available a functional positions on the polymer backbone. In addition, one could take profit of the great stability of the methionine thioeter function in enzymatic ROP conditions to synthesize poly(esters-co-amino acids) which could then be further modified, for example by sulfonation of the thioeter pending group, to obtain polyesters derivatives with better thermal resistance[39]. Then, further works will investigate the polymer functionalization after deprotection and the potential applications of such controlled macromolecular architectures.

Supporting informations of "*Caprolactone-amino acids enzymatic copolymerization: a green route towards new functional copolyesters*"

Containing supplementary characterization of synthesized polymers (TGA curves, *MALDI-ToF spectra* NMR spectra)

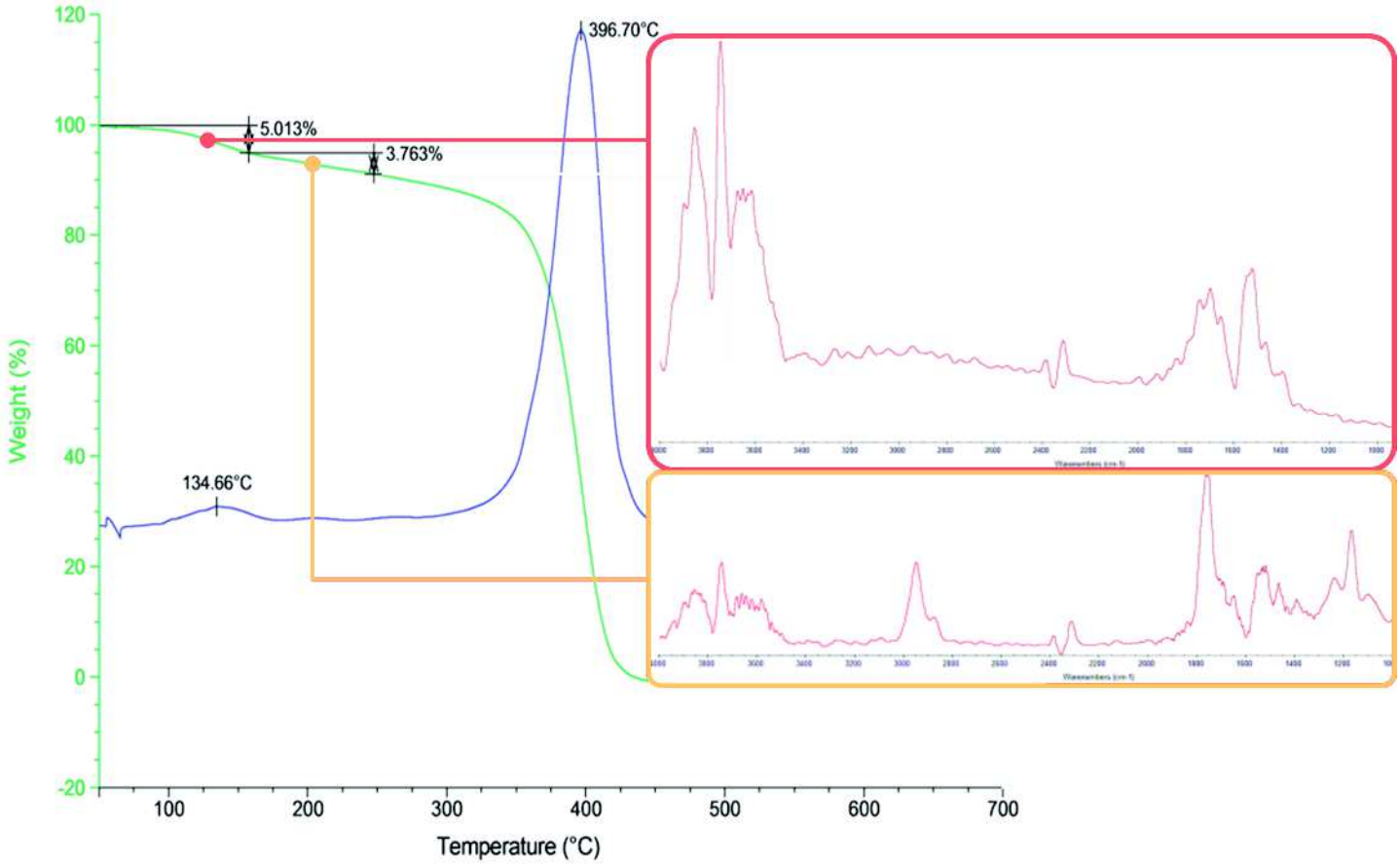


Figure SIV-9. *typical TGA curves of copolymerization product between ε-CL and unprotected amino acid (example of 10 mol% of Met in the feed), in green mass lost, in blue derivative, Red spectrum corresponding to moisture and COV, orange spectrum corresponding to caprolactone*

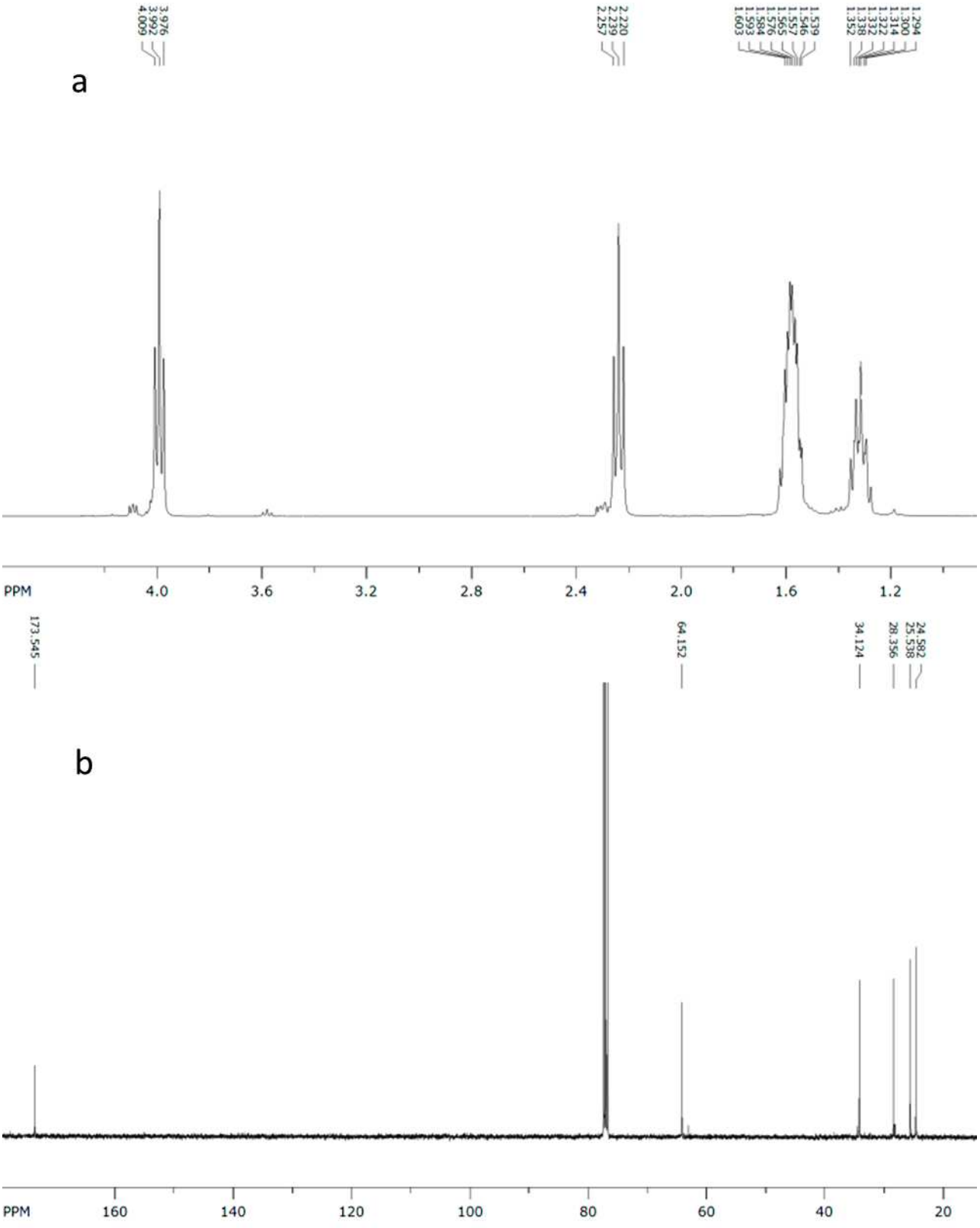


Figure SIV-10 *Typical NMR spectra of copolymerization product between ε-CL and unprotected amino acid (example of 10 mol% of Cys in the feed), a ^{1}H NMR spectrum, b ^{13}C NMR spectrum.*

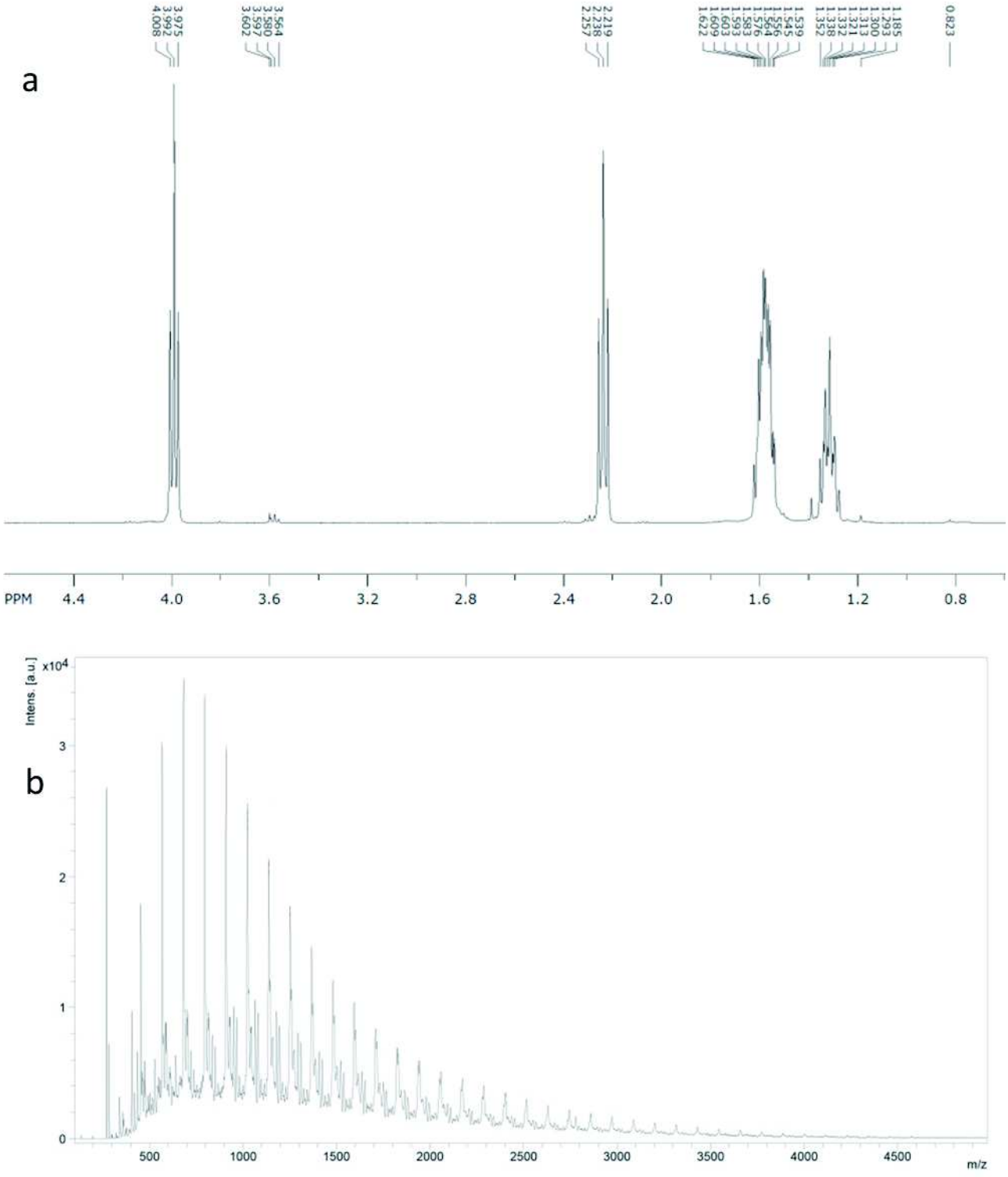


Figure SIV-11. *copolymerization product between ε-CL and 1 mol% of N-Boc Cysteine hexyl ester in the feed; a ^{1}H NMR spectra, b MALDI-ToF spectra*

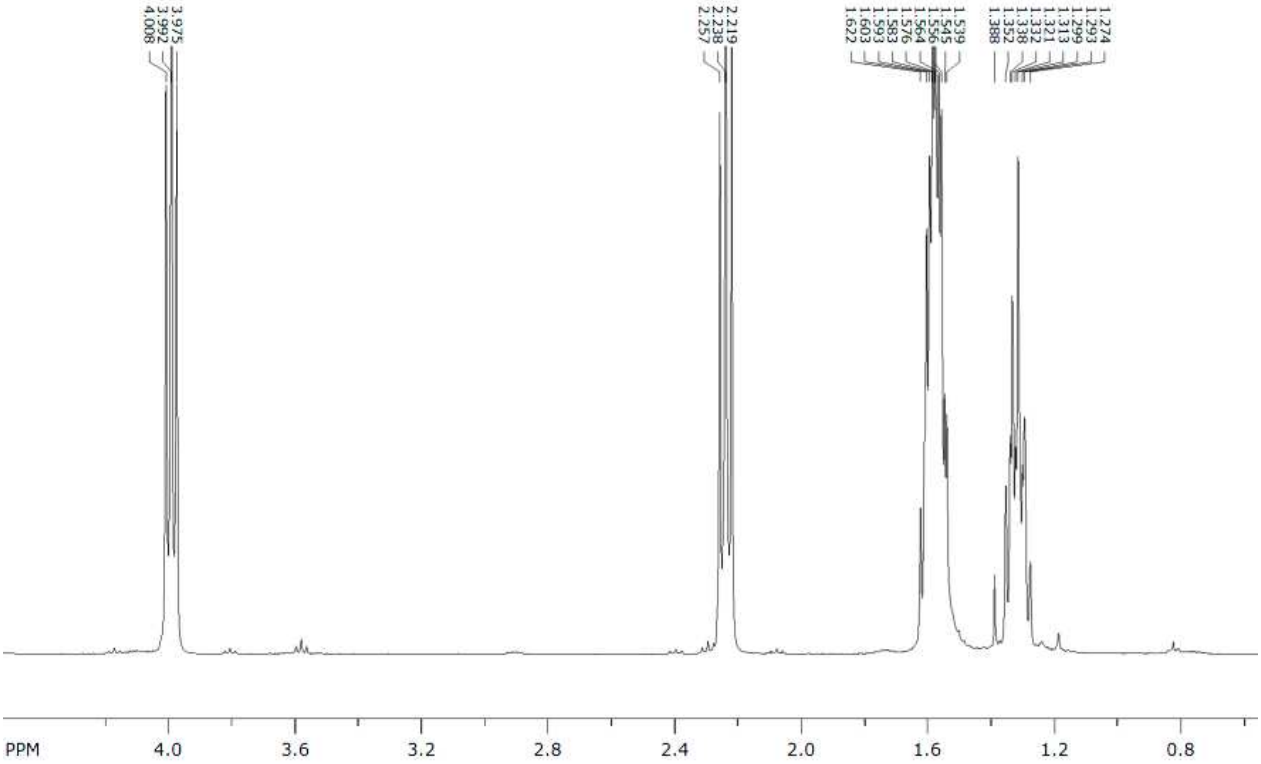


Figure SIV-12. *^{1}H NMR spectra of copolymerization product between ε-CL and 2 mol% of N-Boc Cysteine hexyl ester in the feed*

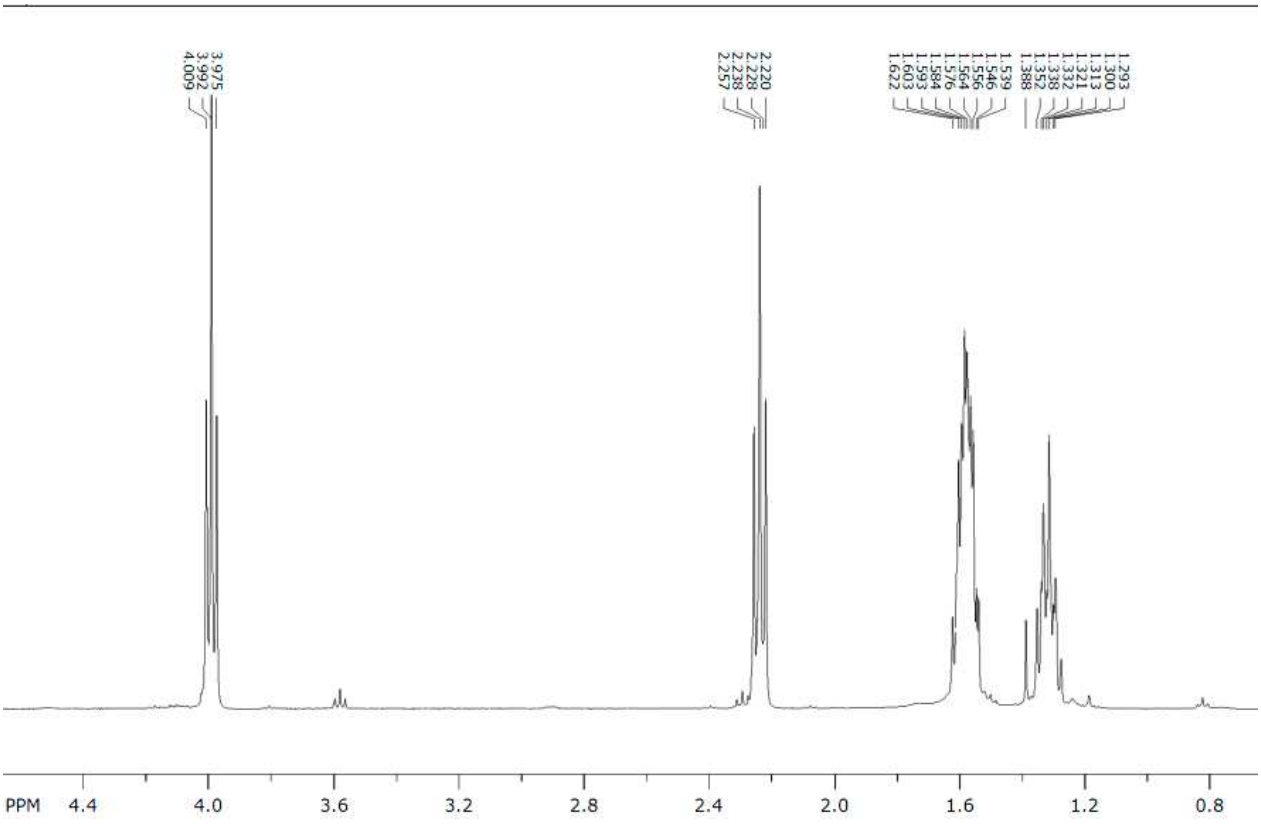


Figure SIV-13. *^{1}H NMR spectra of copolymerization product between ε-CL and 5 mol% of N-Boc Cysteine hexyl ester in the feed*

Conclusion du quatrième chapitre

Ce dernier chapitre nous a permis de montrer la faible réactivité des lactames en eROP en utilisant dans un premier temps, les mêmes enzymes que celles utilisées pour les lactones et dans des conditions similaires. Dans ce cas, aucune conversion du monomère n'a été décelée, ni pour l'anhydride de glycine, ni pour le caprolactame. Dans un deuxième temps nous avons testé un autre type d'enzyme, une protéase, la papaïne, mais en gardant des conditions de réaction en milieu « organique » proches de celles utilisées pour les lactones. Ces conditions nous ont permis d'obtenir une conversion limitée du monomère, environ 5% dans le cas du caprolactame, avec des DPn ne dépassant pas 6 unités par chaine. En revanche, aucune conversion n'a été observée dans ces conditions pour l'anhydride d'acide alpha aminé et ceci probablement du fait de la faible solubilité du monomère dans le solvant. Aussi, nous avons envisagé une troisième approche, en utilisant à nouveau la papaïne, mais en effectuant la réaction non plus en solvant organique (qui favorise la réaction d'addition), mais en condition aqueuse pour permettre une meilleure solubilité des monomères. Une réaction en milieu aqueux pourrait également engendrer une meilleure activité enzymatique de notre protéase car celle-ci, contrairement aux lipases qui sont des enzymes d'interface, ne présente pas une bonne affinité pour les milieux apolaires. Toutefois, cette troisième approche a également produit des résultats mitigés avec, là encore, aucune conversion de l'anhydride de glycine et une conversion très limitée (4%) et un DPn très faible limité à 5 unité maximum dans le cas du caprolactame.

Ces résultats en demi-teinte pour l'eROP des lactames nous ont conduits à envisager une autre approche pour à la fois évaluer l'influence de l'atome d'azote sur cette réaction mais également pour essayer d'incorporer plus de comonomère. Pour cela nous avons envisagé la copolymérisation de caprolactone avec différents acides alpha aminés et, afin de comparer la réactivité des atomes d'azote avec celle de l'atome de soufre, nous avons choisi d'utiliser deux acides aminés soufrés que sont la cystéine et la méthionine.

Ainsi, nous avons expérimenté pour ces acides aminés soufrés deux stratégies de copolymérisation. La première a consisté en l'utilisation de nos deux acides aminés

directement sans modification et la seconde stratégie utilisait la cystéine avec une ou deux de ses fonctions principales protégées.

La copolymérisation avec les acides aminés non protégés n'a pas permis l'incorporation d'acides aminés dans les chaînes de polycaprolactone produites. Cette absence de réaction confirme une fois encore l'importance de la miscibilité des différents co-monomères avec le solvant et entre eux. En effet, même si les lipases sont des enzymes fonctionnant naturellement aux interfaces aqueuse-organique, elles sont efficaces en milieu hétérogène solide-liquide organique. Nous n'avons donc pas été en mesure d'incorporer ces acides aminés peu solubles dans le toluène comme dans la caprolactone. Une solution envisageable pour pallier ce problème serait d'effectuer la synthèse en émulsion avec une phase aqueuse contenant les acides aminés et l'enzyme dispersée, et une phase organique contenant la caprolactone. Toutefois l'eROP en émulsion a montré jusqu'ici des résultats limités avec des limites importantes notamment sur la masse molaire atteignable[44].

Notre seconde approche utilisant une cystéine, dont la fonction amine est protégée par un groupement Boc (*tert*-butoxycarbonyle) et dont la fonction acide est protégée sous forme d'un hexyl ester, nous a permis de synthétiser des chaînes PCL initiées par la cystéine modifiée. Là encore les résultats se sont révélé être mitigés puisque nous n'avons pas été en mesure d'incorporer plus d'une unité cystéine modifiée par chaîne polymère. Toutefois, la caractérisation approfondie des polymères obtenus, par RMN et spectrométrie de masse, a permis de mettre en évidence différentes populations de chaînes polymères. Ceci nous a donc permis d'avancer différents mécanismes d'initiation, soit par l'eau résiduelle du milieu, soit par le thiol de la cystéine modifiée, soit par l'hexanol issu de la transestérification de la cystéine modifiée. De plus, différents mécanismes de terminaison de la réaction ont également pu être proposés tels qu'une terminaison par transestérification (entre 2 chaînes ou avec l'hexyl ester d'une cysteine modifiée), soit par cyclisation. Nos analyses ont également mis en évidence la possibilité d'une substitution de l'hétéroatome de l'initiateur par un oxygène provenant d'une protéine de l'enzyme (ou issu de l'eau de structure de celle-ci).

Même si nous ne sommes pas parvenu à copolymériser ces monomères azotés avec la caprolactone, la synthèse par voie enzymatique de PCL terminé par des acides aminés ouvre

toutefois la possibilité à la synthèse enzymatique d'architectures macromoléculaires plus complexes via des réactions ultérieures sur les groupes fonctionnels disponibles des acides aminés telles que les fonctions thiol de la cystéine ou les fonctions amine (dont le groupe protecteur peut être facilement éliminé) des acides aminés.

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VI. Conclusion générale

En conclusion, nous avons mené une étude sur la polymérisation par ouverture de cycle enzymatique. Ces études apportent des éclairages particuliers et nouveaux sur certaines problématiques de cette réaction. Ainsi les travaux décris dans ce manuscrit permettent de mieux comprendre les mécanismes et les voies d'amélioration de la polymérisation par ouverture de cycle enzymatique. Ce travail s'est articulé autour de quatre chapitres qui intègrent une analyse des travaux antérieurs mais aussi étude de l'influence de différents paramètres liés à ce type de polymérisation (eROP) tels que de la variation de l'hétéroatome intra-cyclique avec l'oxygène, puis le souffre et enfin l'azote. Cette dernière partie, nous a par ailleurs conduits à étudier et développer des aspects assez novateur tel que la copolymérisation de la caprolactone avec divers acides aminés.

La littérature, nous a permis initialement de mettre en évidence des paramètres qui semblent déterminants pour l'eROP d'hétérocycles carbonylés, plus particulièrement pour les lactones et, dans une moindre mesure, pour leurs équivalents lactames et thiolactones.

En premier lieu de ceux-ci, la contrainte de cycle du monomère semble être un facteur important. Si pour la polymérisation chimique des lactones, l'enthalpie de cycle et l'entropie de cycle sont les forces motrices des réactions d'ouverture de cycle, dans le cas de l'eROP des lactones, l'affinité du monomère pour le site actif de l'enzyme ainsi que la stabilité du complexe acyle-enzyme semblent aussi importants. La réactivité d'une lactone semble déterminée par une combinaison de ces trois facteurs. Ainsi, pour les cycles à moins de 5 chainons, le gain d'entropie reste une force motrice suffisante, tandis que la réactivité augmente à partir des cycles à 6 chainons jusqu'aux cycles à 10 chainons, pour lesquels la contrainte de cycle est quasiment nulle.

La morphologie de l'enzyme est également un facteur important, et c'est sans surprise que la littérature a montré la prédominance d'un nombre restreint d'enzymes permettant l'eROP

de lactones, de lactames ou de thiolactones. Parmi les enzymes les plus efficaces, celles dont la morphologie a été étudiée en détail ont en commun une structure de site actif en forme d'entonnoir. Ainsi, dans cette étude nous avons principalement utilisé des enzymes ayant cette particularité, à savoir, la lipase B de *Candida antarctica* et la lipase de *Burkholderia cepacia*.

La bibliographie a aussi montré que certains paramètres, comme l'influence de l'hétéroatome intra-cyclique, n'avaient pas été étudiés et que des contradictions subsistent quant à la nature du milieu et aux conditions les plus favorables. En effet, plusieurs auteurs ont étudié l'influence du solvant mais parviennent à des résultats contradictoires. Cela peut être dû au fait que ces réactions sont complexes et que, même si l'affinité du solvant pour l'enzyme est importante, la solubilité du monomère, de l'oligomère et du polymère sont également des paramètres déterminants qui ont été très souvent ignorés dans ces études.

Notre seconde partie, centrée sur l'étude de l'eROP des lactones et plus spécifiquement du lactide, a permis de confirmer certaines des hypothèses précédemment formulées. Nos résultats ont ainsi démontré l'influence de la contrainte de cycle, la non-réactivité de la γ-butyrolactone (cycle à cinq chainons), mais également la réactivité très réduite d'enzymes ayant un site actif en forme de tunnel telle que la lipase de *Candida rugosa*. Ces travaux ont également mis en évidence l'influence de l'hydratation du milieu grâce à une étude sur différentes méthodes de séchage de l'enzyme et du milieu réactionnel. Nous avons ainsi pu constater la grande influence de l'eau d'hydratation de l'enzyme et de l'importance d'en minimiser la teneur pour améliorer la taille des polymères synthétisés. Ainsi, la lyophilisation nous a permis de réduire l'eau d'hydratation de nos enzymes de 30% à 80% et d'augmenter la longueur des chaînes de 20% à 40% pour respectivement N435 et LBC.

Ce second chapitre nous a également permis d'améliorer la cinétique de polymérisation du lactide. En effet, nos essais sur l'influence du solvant ont montré que l'ajout de triéthylamine comme co-solvant permettait, en contrepartie d'une racémisation partielle, d'accroitre considérablement la cinétique de polymérisation enzymatique du lactide. Cet ajout de co-solvant a une nette influence sur la structure des polymères formés, avec la formation de deux populations distinctes de chaînes polymères, une première population similaire à celle formée sans co-solvant et une seconde de masse molaire plus importante, selon des

caractérisations faites par chromatographie d'exclusion stérique mais également par spectroscopie de masse.

Enfin, ces résultats nous ont permis de formuler certaines hypothèses mécanistiques sur l'eROP et sur les mécanismes possibles d'assistance de la triéthylamine qui pourraient relever de (i) l'activation nucléophile du lactide, (ii) l'activation basique de la serine de la lipase ou de (iii) l'activation basique des alcools de fin de chaîne des polymères.

Le troisième chapitre, centré quant à lui sur la polymérisation des thiolactones, a permis de mieux appréhender l'influence de l'hétéroatome intra-cyclique sur l'eROP. Tout d'abord concernant la réactivité, nous avons montré que la thiocaprolactone avait une réactivité moindre que la lactone correspondante, aussi bien en termes de conversion, avec une diminution de l'ordre de 25%, qu'en termes de masse molaire finale avec un Mn divisé par 5. Même si cela demande encore des études complémentaires, il est probable que ce constat puisse être étendu aux autres thiolactones par rapport aux lactones correspondantes.

Dans cette partie sur l'influence de l'atome de soufre, nous avons également étudié différentes stratégies de copolymérisation entre la caprolactone et la thiocaprolactone dans le but de produire des copolymères de différentes structures. Ces expérimentations ont donné des résultats inattendus mais intéressants, notamment en ce qui concerne le mécanisme de copolymérisation. Ainsi, la première stratégie, consistant en la polymérisation directe du mélange des deux monomères, a montré l'obtention de chaînes polymères avec une structuration très particulière. En effet, les analyses de spectrométrie de masse semblent mettre en évidence des enchainements successifs de trimères de chacun des deux co-monomères. Cette structuration inattendue pourrait être due à un mécanisme de polymérisation par étapes avec, dans un premier temps une trimérisation de chaque co-monomère puis une polymérisation de ces trimères. La seconde stratégie, basée sur une copolymérisation en deux étapes avec d'abord la synthèse d'homopolymère puis ajout dans un deuxième temps du second monomère, a quant à elle montré la possibilité de former des copolymères ayant une structure plutôt à bloc. Ceci semble indiquer que, même si elles sont présentes, les réactions de trans-estérification et de trans-thio-estérification restent relativement limitées dans ces conditions.

Afin de compléter notre étude sur l'hétéroatome intra-cyclique dans l'eROP, nous nous sommes intéressés à la substitution de l'oxygène intra-cyclique par un atome d'azote dans le quatrième chapitre. Dans cette dernière partie de notre étude, nous avons pu constater l'extrême difficulté de polymériser des lactames par voie enzymatique. En effet, ces hétérocycles sont extrêmement stables et nous n'avons pas été en mesure de réaliser de manière significative l'ouverture et la polymérisation des lactames. Les conversions restent inférieures à 10% et les degrés de polymérisation ne dépassent pas 4 unités.

Afin de poursuivre et compléter notre étude de l'influence des hétéroatomes sur la polymérisation enzymatique, nous avons donc envisagé de manière assez innovante, la copolymérisation de la caprolactone avec des acides aminés tels que la cystéine et la méthionine. Ces acides aminés soufrés ont été choisis afin d'avoir un point de comparaison avec les monomères cycliques soufrés étudiés précédemment mais également afin d'obtenir des copolymères permettant d'envisager des fonctionnalisations particulières, des réticulations ultérieures …

Pour ces copolymérisations nous avons envisagé trois formes différentes pour nos acides aminés :

- La première consiste à l'utiliser sous sa forme naturelle,
- la seconde est celle où la fonction amine est protégée par un groupement BOC,
- la troisième présente également une fonction amine protégée par un BOC mais la fonction acide est estérifiée afin d'accroitre sa réactivité vis-à-vis de nos enzymes.

La polymérisation avec les acides aminés sous leurs formes natives s'est révélée quasiment impossible très probablement à cause de problèmes de solubilité des monomères mais également à cause de leur forte polarité qui semble engendrer une faible affinité avec le site actif de l'enzyme.

Dans le cas des acides aminés protégés, les résultats ont montré une homopolymérisation de caprolactone initiée par la fonction thiol de l'acide aminé. En revanche il n'y a pas ou peu d'insertions d'acides aminés dans la chaîne qui auraient pu se produire par réactions de trans-estérification impliquant la fonction acide carboxylique des acides aminés.

Enfin, en ce qui concerne la troisième forme de l'acide aminé (fonction acide estérifiée), on observe que la population majoritaire est constituée de chaînes caprolactone terminées par un acide aminé résultant de réactions de trans-estérification impliquant l'hexyl ester.

Tous ces résultats nous ont toutefois permis de recueillir des informations importantes quant à la réactivité relative des fonctions thiol, amine et ester vis-à-vis de la polymérisation enzymatique. Parmi les conclusions qui peuvent être tirées, on peut dire que la présence d'un ester d'acide gras favorise la polymérisation enzymatique. Cette étude met également en avant la moins bonne réactivité de la fonction thiol comparée à l'hydroxyle. Toutefois, ces résultats ne nous permettent pas de conclure quant à la réactivité des fonctions amines par rapport aux autres fonctions.

L'analyse détaillée de ces résultats de copolymérisation avec des acides aminés nous a également apporté des informations intéressantes relatives au mécanisme de polymérisation enzymatique. En effet, nous avons pu observer en spectrométrie de masse la présence d'une population de chaînes polymères initiées par une serine protégée N-BoC ce qui nous indique que durant la réaction d'estérification catalysée par les lipases, l'alcool de l'amorceur est susceptible d'être échangé avec celui de la serine du site actif. Cette dernière conclusion est tout à fait compatible avec les mécanismes réactionnels proposés jusqu'ici dans la littérature néanmoins son observation dans une telle proportion reste relativement surprenante et inédite.

Cette étude apporte donc globalement des éclairages particuliers et novateurs sur certaines zones d'ombres qui subsistaient concernant l'eROP. Même si nos résultats apportent de nouvelles interrogations, ils posent néanmoins une nouvelle base de recherche utile pour de futurs travaux permettant de faire avancer progressivement ce pan particulier dans le domaine de la catalyse des polymères, qui fait l'objet dans le monde de très nombreux investissements au vue des challenges soulevés et des grandes mutations attendues.

Perspectives de cette étude

Ces résultats ouvrent de nombreuses perspectives pour la synthèse enzymatique de polymères et également, dans une moindre mesure, pour l'utilisation des enzymes dans les réactions de synthèse chimique plus traditionnelles.

L'identification de paramètres clés, mis en évidence ouvre déjà des voies simples et directes pour :

(i) la présélection des enzymes potentiellement actives en fonction de la structure de leur site actif,

(ii) l'optimisation des conditions réactionnelles en combinant la solubilité du monomère et l'affinité de l'enzyme pour le solvant.

L'ajout d'un co-solvant actif lors de la polymérisation du lactide ouvre également des possibilités intéressantes pour d'autres monomères en vue d'améliorer la cinétique de réaction et la rendre ainsi plus intéressante et compétitive dans un contexte d'application industrielle, à grande échelle.

De même, la possibilité de contrôler la microstructure de copolymères formés par eROP permet d'envisager un large champ d'investigations pour de nouvelles architectures macromoléculaires et de riches développements particulièrement dans le domaine biomédical. Par ailleurs, la possibilité d'une post-fonctionnalisation simple des chaînes de PCL grâce à la copolymérisation avec des acides aminés naturels permet d'ouvrir des horizons intéressants dans ces mêmes domaines que sont les polymères fonctionnels à architecture contrôlée et leur application dans le secteur biomédical.

Ensuite, en ce qui concerne les mécanismes réactionnels, malgré les conclusions qui ont pu être avancées, nos travaux ont également mis en évidence un certain nombre de lacunes dans la compréhension globale de l'eROP. Ceci ouvre donc naturellement la porte à de nombreux travaux à venir dans le domaine de la modélisation moléculaire pour mieux appréhender les phénomènes d'activation par le solvant ainsi que les phénomènes de solvatation partielle, afin de comprendre en détail ce qui ce passe à l'interface entre le catalyseur et le milieu réactionnel. Ces travaux posent également de nouvelles questions

relatives au mécanisme de ROP et de trans-estérification catalysées par les enzymes, notamment en ce qui concerne les phénomènes de substitution qui peuvent avoir lieu dans le site actif et qui sont encore très mal connus. Enfin, la micro-structuration particulière observée lors de la copolymérisation avec la thiocaprolactone a montré un certain manque de connaissances et de compréhension de la réactivité relative des différents oligomères en fonction de leurs degrés de polymérisation. Aussi, une meilleure compréhension des règles reliant la réactivité des oligomères et chaînes en croissance à leur degré de polymérisation permettrait certainement des avancées majeures pour la synthèse enzymatique de polymères à la microstructure parfaitement contrôlée.

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Annexe 1 - Les douze principes de la Chimie Verte

1. La prévention de la pollution à la source en évitant la production de résidus.
2. L'économie d'atomes et d'étapes qui permet de réaliser, à moindre coût l'incorporation de fonctionnalités dans les produits recherchés tout en limitant les problèmes de séparation et de purification.
3. La conception de synthèses moins dangereuses grâce à l'utilisation de conditions douces et la préparation de produits peu ou pas toxiques pour l'homme et l'environnement.
4. La conception de produits chimiques moins toxiques avec la mise au point de molécules plus sélectives et non toxiques impliquant des progrès dans les domaines de la formulation et de la vectorisation des principes actifs et des études toxicologiques à l'échelle cellulaire et au niveau de l'organisme.
5. La recherche d'alternatives aux solvants polluants et aux auxiliaires de synthèse.
6. La limitation des dépenses énergétiques avec la mise au point de nouveaux matériaux pour le stockage de l'énergie et la recherche de nouvelles sources d'énergie à faible teneur en carbone.
7. L'utilisation de ressources renouvelables à la place des produits fossiles. Les analyses économiques montrent que les produits issus de la biomasse représentent 5 % des ventes globales de produits chimiques et pourraient atteindre 10 à 20 % en 2010. Plus de 75% de l'industrie chimique globale aurait alors pour origine des ressources renouvelables.
8. La réduction du nombre de dérivés en minimisant l'utilisation de groupes protecteurs ou auxiliaires
9. L'utilisation des procédés catalytiques de préférence aux procédés stœchiométriques avec la recherche de nouveaux réactifs plus efficaces et minimisant les risques en terme de manipulation et de toxicité. La modélisation des mécanismes par les méthodes de la chimie théorique doit permettre d'identifier les systèmes les plus efficaces à mettre en œuvre (incluant de nouveaux catalyseurs chimiques, enzymatiques et/ou microbiologiques).
10. La conception des produits en vue de leur dégradation finale dans des conditions naturelles ou forcées de manière à minimiser l'incidence sur l'environnement.
11. La mise au point des méthodologies d'analyses en temps réel pour prévenir la pollution, en contrôlant le suivi des réactions chimiques. Le maintien de la qualité de l'environnement implique une capacité à détecter et si possible à quantifier, la présence d'agents chimiques et biologiques réputés toxiques à l'état de traces (échantillonnage, traitement et séparation, détection, quantification).
12. Le développement d'une chimie fondamentalement plus sûre pour prévenir les accidents, explosions, incendies et émissions de composés dangereux.

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Stéphane DUCHIRON

Etude de la polymérisation enzymatique de monomères hétérocyclocarbonyliques

Résumé

Le domaine des polymères biosourcés connait une croissance rapide mais se pose toujours le problème d'une synthèse plus respectueuse de l'environnement. En cela, la catalyse enzymatique est une voie prometteuse. Ce travail vise donc à étudier et comprendre la polymérisation enzymatique par ouverture de cycle (eROP) afin d'en dépasser les limitations qui sont principalement : une cinétique lente, une faible masse molaire des polymères obtenus ou une variété limitée des fonctions chimiques polymérisables. La première partie de notre étude, portant sur les lactones, a mis en évidence la possibilité d'activer la réaction via une amine tertiaire. La seconde, qui traite des thiolactones, a mis en exergue des mécanismes spécifiques de copolymérisation avec plusieurs étapes de croissance de chaînes. Enfin, nous avons synthétisé des polyesters porteurs d'acides aminés, ouvrant ainsi la voie à des polymères « fonctionnalisables » et à de nouvelles architectures macromoléculaires.

Mots clefs : catalyse, enzymes, lipase, lactone, thiolactone, acide aminé, polymérisation par ouverture de cycle

Abstract

The field of biobased polymers is experiencing a rapid growth but the development of more environmentally friendly synthesis methods remains a problem. With this in mind, enzymatic catalysis is a promising tool but it has some limitations such as slow polymerization kinetics, the low molecular weight of the produced polymers and a limited range of polymerizable monomers. This work aims at a better understanding of the enzymatic ring opening polymerization reactions (eROP) with a view to overcoming these limitations. To achieve this, three main topics have been investigated. The first, focusing on lactones, has demonstrated the possibility of activating the polymerization reaction with a tertiary amine. The second part, dealing with thiolactones as sulfur-based monomers, highlighted specific copolymerization mechanisms comprising of several distinct steps of polymer chain growth. Finally, polyesters bearing amino acids were synthesized, thus paving the way for functionalizable polymers and new macromolecular architectures.

Keywords: catalysis, enzymes, lipase, lactone, thiolactone, amino acid, ring opening polymerization