Université de Montréal

The Use of *Candida antarctica* Lipase B for the Synthesis of Macrocycles and Polymers Based on Natural Products

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Résumé

Les matériaux utilisés pour les applications biomédicales doivent être biocompatibles, et idéalement biodégradables. Les acides biliaires proviennent de sources naturelles et sont présents dans le corps humain. De plus, les polyetsers composés en partie de ces molécules possèdent des liens hydrolysables, une mémoire de forme thermique, et leur flexibilité peut être variée. Jusqu'à présent, la synthèse de ces matériaux exigeait l'utilisation de catalyseurs contenant des métaux de transition lourds pour l'étape de macrocyclisation. Puisque la polymérisation par ouverture de cycle nécessite des précurseurs cycliques, l'étape de lactonisation fut réalisée par voie enzymatique, au lieu d'utiliser des catalyseurs à plus grande toxicité. De plus, une seule étape enzymatique a pu remplacer deux étapes de synthèse organique, avec un rendement de 58 % et l'obtention d'un matériel transparent. Ces macrocycles nouvellement obtenus ont par la suite été polymérisés par ouverture de cycle, de façon similaire à la technique élaborée par notre groupe en 2013, tout en optimisant la durée de réaction. En 15 heures, une masse molaire relativement grande de 40 000 g/mol fut obtenue, tout en maintenant la dispersité sous 1.4 et la température de transition vitreuse à 12 °C. Pour valider le principe de cyclisation et de polymérisation enzymatique, les conditions optimales pour combiner l'acide thapsique et le 1,10-decanediol furent préalablement déterminées. Entre autres, la durée de réaction et la quantité d'enzyme nécessaire furent analysées. Les polymères semi-crystallins obtenus possèdent aussi de grandes masses molaires et de basses dispersités. Or, il est possible d'utiliser un enzyme à la fois pour la fermeture et pour l'ouverture de cycle de molécules rigides à cœur stéroïdal, telles que les acides biliaires. Cette synthèse permet la production de matériaux plus biocompatibles, tout en favorisant plusieurs principes de chimie verte.

Mots-clés : Lipases, Lactonisation, Polymérisation par Ouverture de Cycle, Acides Biliaires, Chimie Verte

Abstract

Materials used in biomedical applications need to be biocompatible and ideally biodegradable. Bile acids are natural occurring compounds found in humans, and their polyesters possess hydrolyzable bonds, thermal shape memory and tunable flexibility. Until now, the synthetic pathway to obtain such materials required transition metal catalysts for the macrocyclization step, which is necessary to perform ring-opening polymerization (ROP). To circumvent the need for such catalysts, enzymatic ring closing was performed using lipases. Conveniently, two synthetic steps were replaced with a single step, using a renewable and reusable catalyst, with 58 % yield and a colorless product. The bile acid-containing macrocycles were then enzymatically polymerized as described in our previous work, while optimizing the reaction time. In 15 hours, relatively high M_w of 40 000 g/mol were obtained, while maintaining the dispersity ≤ 1.4 and a glass transition temperature of about 12 °C. As a proof-of-concept, conditions for the enzymatic ring closure of thapsic acid with 1,10-decanediol were determined beforehand. While optimizing for enzyme amount and reaction time, enzymatic ROP conditions to obtain di- and tetralactones from these monomers were established. The resulting semi-crystalline polymers also possess relatively high molecular weight and low dispersity. Hence, the use of lipases for both ring-closing and ring-opening reactions now shows potential for large, rigid moieties in addition to more mobile structures, using the same enzyme. This is a step towards the production of more biocompatible polymers, with a synthetic pathway that follows many green chemistry principles.

Keywords : Lipases, Lactonization, Ring-Opening Polymerization, Bile Acids, Green Chemistry

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List of Acronyms, Symbols and Abbreviations

ATRP: Atom-transfer radical polymerization

BAs: Bile acids

BMA: n-butyl methacrylate

CA: Cholic acid

CAEG: Cholic acid-ethylene glycol

CALB: *Candida antarctica* lipase B

CRL: *Candida rugosa* lipase

DCC: N,N'-dicyclohexylcarbodiimide

DCU: Dicyclohexylurea

$Đ_M$: Dispersity

DMAP: 4-dimethylaminopyridine

DMATC: 2-dimethylaminotrimethylene carbonate

DO: *p*-dioxanone

DP_n: Degree of polymerization

DSC: Differential scanning calorimetry

eARTP: Enzymatic atom-transfer radical polymerization

ED-ROMP: Entropy-driven ring-opening metathesis polymerization

ED: Entropy driven

EM: Effective molarity

eRC: Enzymatic ring closing

eROP: Enzymatic ring-opening polymerization

ESI: Electrospray ionization

GL: Glycolide

HPLC: High-performance liquid chromatography

iCALB: Immobilized *Candida antarctica* lipase B

ITC: (5S,6S)-dimethyl 5,6-O-isopropylidene-1,3-dioxepin-2-one

LC-MS: Liquid chromatography-mass spectrometry

LCA: Lithocholic acid

Log P: Logarithm of the partition coefficient, defines degree of polarity

MDI: 4,4'-methylenebis(phenyl isocyanate)

Me: Methyl

MMA: Methyl methacrylate

mmHg: Torr

MS: Mass spectrometry

MWR: Microwave radiation

NMR: Nuclear magnetic resonance

OC: 2-oxo-12-crown-4-ether

PBS: Polybutylene succinate

PCL: Polycaprolactone

PDL: ω-pentadecanolide

PEG: Polyethylene glycol

PF: *Pseudomonas fluorescens*

PHAs: Poly(hydroxyalkanoate)

PHEA: Poly(hydroxyethyl-L-asparagine)

PLLA: Poly(L-lactide)

PLU: Propyl laurate units

PPDL: Poly(pentadecanolide)

PPL: Porcine pancreatic lipase

PS: *Pseudomonas cepacia*

RCM: Ring-closing metathesis

RML: *Rhizomucor miehei* lipase

ROMP: Ring-opening metathesis polymerization

ROP: Ring-opening polymerization

RT: Reaction time

SEC: Size exclusion chromatography

TA: Thapsic acid

T_c: Crystallization temperature

T_d: Degradation temperature

T_g: Glass transition temperature

TGA: Thermogravimetric analysis

T_m: Melting temperature

TSAS: Transition state analog substrate

δ-VL: δ-valerolactone

ε-CL: ε-caprolactone

Things work out best for those who make the best of how things work out.

- John Wooden

Acknowledgments

Pursuing this project would not have been possible without the tremendous support I benefit from every day. I am incredibly lucky to have been born in an environment where opportunities multiply. My parents have always made sure I would benefit from the best, while letting me earn my success. I am eternally grateful for the values passed on, which make me whom I am.

James, the love of my life, is the one who never questioned my determination. He pushed me into realizing my dreams, even when I doubted myself. He can be proud of this achievement, because it is partly his. He was present at *every* moment, the good and the bad. He is responsible for the confidence I gained throughout these years, as a person and as a scientist.

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I also gratefully acknowledge the comity taking the time to evaluate this work. I welcome your feedback.

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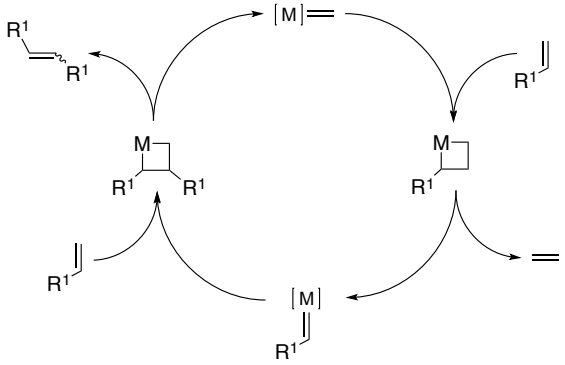
Chapter 1 - Introduction

1.1 Metathesis reactions

In the late 20th century, a very important type of catalyst was engineered: one that could catalytically allow carbon-carbon double bond cleavage and formation. These are the Grubbs and Schrock catalysts, which won the 2005 Nobel Prize of chemistry along with Yves Chauvin for the discovery of the underlying mechanism. The stability of these catalysts enabled their use in organic chemistry, in both protic and aprotic solvents, which allowed a whole new array of possibilities in both small molecule, and polymer synthesis fields,[1] such as ring-closing metathesis (RCM), and ring-opening metathesis polymerization (ROMP).

The mechanism of metathesis, discovered in 1971, is as follows: the metal carbide acts as the catalyst to metathesize the two terminal alkenes into an internal one, and ethylene as the side product. It is the removal of this side-product that drives the reaction to completion (Scheme 1.1).[2]



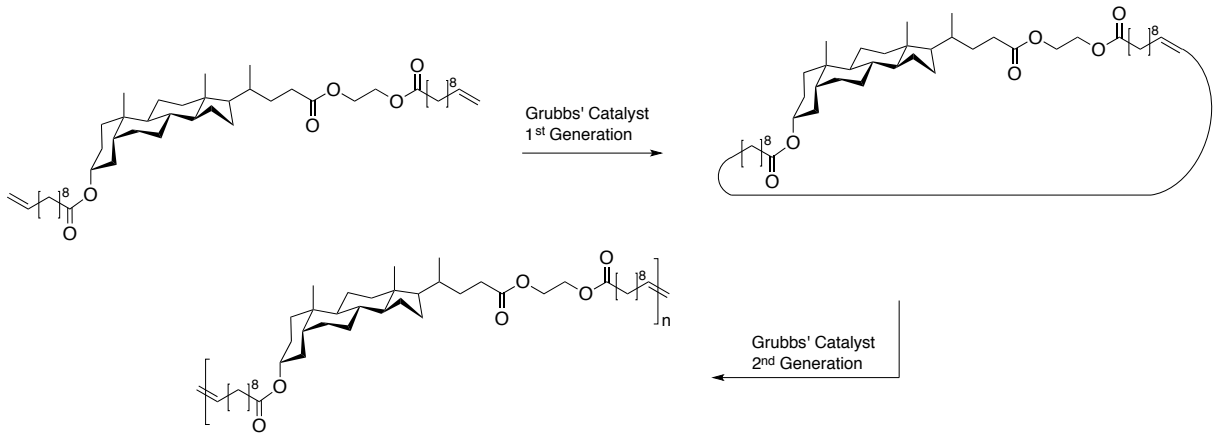
Scheme 1.1 Metathesis catalytic cycle rationalized by Yves Chauvin in 1971.

It is the efforts of Prof. Chauvin that led researchers such as Schrock and Grubbs to engineer the new catalysts. While Prof. Schrock developed a whole family of molybdenum- and tungsten-alkylidene complexes that are known as the most active alkene metathesis catalysts, Prof. Grubbs made use of the ruthenium element. Indeed, in 1992, Grubbs and co-workers reported the "first molecularly well-defined ruthenium-carbene complex that was not only active towards polymerization of norborene but was also stable in the presence of protic solvents."[3] Since Grubbs' catalysts are known to accommodate a multitude of functional groups and to be tolerant to standard organic chemistry techniques, they gained much attention.

Prof. Zhu's research group used Grubbs' catalysts to perform RCM and ROMP on naturally occurring bulky substrates, such as bile acids. Indeed the group's research aims towards the use of bile acids and other natural compounds when possible. Projects such as supramolecular hydrogels,[4-5] dental composites,[6-7] drug-delivery polymers,[8] shape memory polymers,[9] and thermosensitive molecular necklaces[10] all make use of bile acids. Indeed, these bioavailable molecules are particularly interesting for their functonalizable, bent, rigid structure that allows fine-tuning of thermo-mechanical properties of final products.

A demonstration of the versatility of Grubbs' catalysts was published in 2006 by Gautrot and Zhu. They successfully synthesized bile acid-based degradable elastomers using the Grubbs' first generation catalyst for RCM and the second generation catalyst for ROMP (Scheme 1.2).[9, 11-12] This unveiled a large array of possibilities in the polymer synthesis industry, since new kinds of naturally-derived compounds, possessing bulky and rigid structures, were efficiently polymerized. The high molecular weight (M_w, up to 452 000

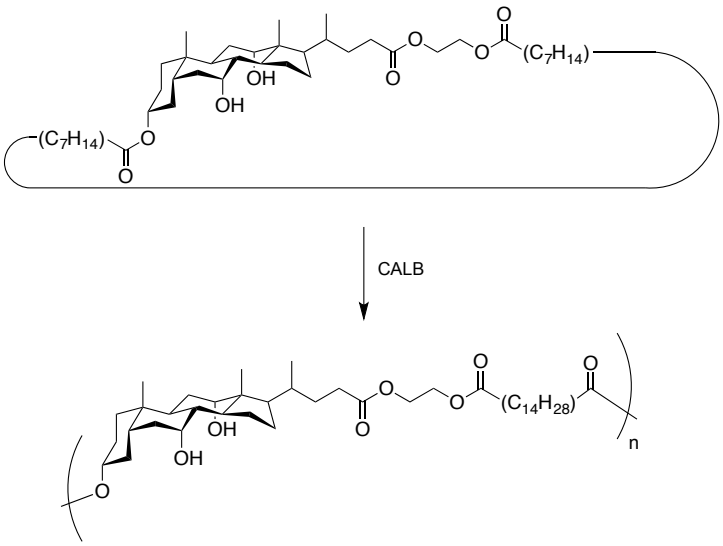
g/mol), degradable, and flexible materials showed great potential in the biomedical field, being bio-based and possessing many functional sites.



Scheme 1.2 RCM and ROMP of bile acid-based molecules using two generations of the Grubbs' catalyst.

1.2 Enzymatic alternative

The greatest drawback of using transition metal catalysts is the contamination they cause. Indeed, they are difficult to remove from the final product and they are not desired for applications in medicine. Hence, in 2013, Strandman et al. developed a new pathway for the ring-opening polymerization (ROP) step, greatly diminishing the final ruthenium content in the polymers (Scheme 1.3).[13] This was done by exploiting the particularly impressive specificity and robustness of lipases, which gave polymers with M_w of up to 30 400 g/mol. Indeed, instead of using ROMP, the ability of lipases to selectively hydrolyze ester bonds was brought into play.



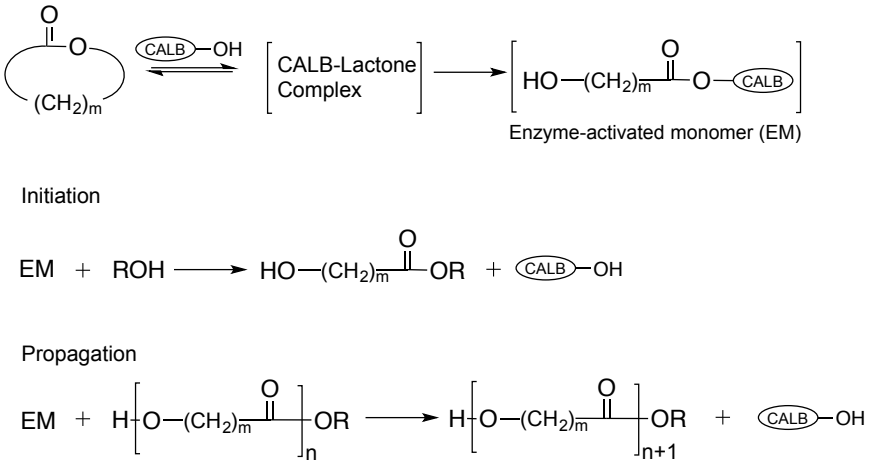
Scheme 1.3 Enzyme-catalyzed ROP of bile acid-based molecules.

Lipases have been used to catalyze esterification reactions for several decades now. Researchers use them for the advantageous specificity, renewability, availability and sustainability they have to offer.[14] Indeed, there are many species of lipases and each of them has a different degree of compatibility with substrates, which makes their use applicable to almost any molecule.[15] Nevertheless, enzymes catalyze reactions at specific amino acid sites, which minimizes the quantity of resulting side products, rendering the process greener, since less purification steps are required. Immobilizing the enzymes also allows their reuse and helps to accommodate larger substrates, such as bile acids, opening the funnel leading to the active site.[16] Finally, most lipases are known to withstand anhydrous conditions and relatively high temperatures, compared to most enzymes that function at body temperature.[17-18]

While the high specificity of enzymes is an advantage, it can also be a drawback, not allowing all molecules to be compatible with this kind of reaction. Furthermore, lipases

possess a limited robustness, since temperatures around 100 °C and higher will deactivate them. Although relatively large M_w are obtained when using enzymes as a catalyst for ROP, they are still significantly lower (around 10-fold) compared with the polymers obtained when Grubbs' catalysts are used.[9, 13, 19] Nevertheless, enzymatic synthesis continues to amaze researchers, as will be discussed in the next chapters of this memoir.

The eROP of lactones happens via an enzyme-activated monomer (EM) at the serine residue of the lipase's active site. As seen in Scheme 1.4, the coupling of lipase with the lactones is the rate-determining step, since every propagation step involves an EM. Furthermore, the propagation species, ω-hydrocarboxylic acid, is generated by a nucleophilic attack, usually from water contained in proximity of the enzyme or from a voluntarily added nucleophile.[20-21] A very similar mechanism happens in the case of lactonization, as both reactions involve esterification or transesterification reactions. More details will be discussed throughout this memoir.



Scheme 1.4. General mechanism of lipase-catalyzed ROP, as explained by Uyama et al.[21]

1.3. Topics covered in this memoir

This work aims to contribute to the field of polymer and bioorganic chemistry by facilitating the pathway to macrocyclic molecules and their resulting polymeric materials. With the objective of obtaining completely heavy transition metal-free, biodegradable, and biocompatible polymers, a pathway was elaborated to replace the RCM step by a more bio-friendly procedure. In addition, rendering syntheses as 'green' as possible is a constant ambition in Prof. Zhu's research group, and it is also one of the main focuses of this work.

Chapter 2 of this memoir is a published literature review (Champagne, E.; Strandman, S.; Zhu, X.-X., *Recent developments and optimization of lipase-catalyzed lactone formation and ring-opening polymerization*. Macromolecular Rapid Communications, **2016**), and serves as a complete introduction to the field of enzyme-catalyzed lactonization and polymerization. Key parameters to consider when designing new enzyme-catalyzed lactonization pathways are thoroughly discussed, while recent advances in eROP are explored. This review was almost entirely written by É. Champagne. Dr. Strandman contributed to this paper by giving her expert advice in the polymerization field, and Prof. Zhu helped with the selection of contents and final presentation style.

Chapter 3 of this memoir is an accepted version of a full paper (Champagne, E.; Lévaray, N.; Zhu, X. X., *A two-step enzymatic synthesis of biocompatible polymers made from cholic acid*. ACS Sustainable Chemistry & Engineering, **2016**) and relates the most important results obtained during this study. Indeed, two kinds of molecules were macrocyclized using CALB, and they were subsequently polymerized using the same enzyme, completely eliminating the need for transition metal catalysts to produce bile acid-based biodegradable polyesters. E. Champagne is responsible for the research, laboratory work, and writing of this article. N.

Levaray helped with NMR analysis and gave his expert advice in organic chemistry. Prof. Zhu directed the study and helped format the final version of the article.

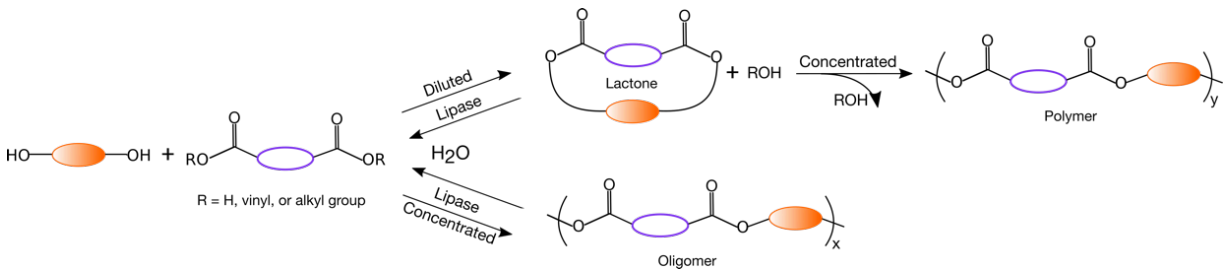
Chapter 2 - Recent Developments and Optimization of Lipase-Catalyzed Lactone Formation and Ring-Opening Polymerization[†]

Review

[†] Included with permission. (22) Champagne, E.; Strandman, S.; Zhu, X.-X., *Recent developments and optimization of lipase-catalyzed lactone formation and ring-opening polymerization*. Macromol. Rapid Commun. **2016**.

2.1 Abstract

To obtain materials useful for the biomedical field, toxic catalysts should be removed from the synthetic route of polymerization reactions and of their precursors. Lipase-catalyzed ring-opening polymerization and the synthesis of cyclic precursors can be performed with the same catalyst under different conditions. In this review, we highlight the use of lipases as catalysts and optimization of their performance for both ring-closing and ring-opening polymerization, via varying parameters such as ring size, concentration, substrate molar ratio, temperature, and solvent. While the conditions for ring-closing reactions and ring-opening polymerizations of small molecules, such as ε-caprolactone, have been extensively explored using *Candida antarctica* lipase B (CALB), the optimization of macrocyclization especially for more bulky substrates is surveyed here. Finally, recent methods and polymer architectures are summarized with an emphasis on new procedures for more sustainable chemistry, such as the use of ionic liquids as solvents and recycling of polyesters by enzymatic pathways.



2.2 Introduction

Enzymes are the tools of nature. They are essential to all living organisms, responsible for metabolic and digestive functions. As catalysts, enzymes can render the synthetic processes greener: they allow milder reaction conditions, circumvent the use of toxic organometallic catalysts and improve the specificity of reactions, can be readily produced in large scale, and are often reusable for several synthetic cycles in their immobilized form, which in turn can improve the cost-efficiency of industrial processes.[23-24] The first investigations on the activity of a specific class of enzymes, lipases, have been reported as early as in 1898,[25] and enzymatic polymerization methods have been developed since mid-1980s.[26-28] Since then, enzymes have become an important part of the toolkit of synthetic chemists and have gained increasing popularity as 'green' catalysts in macromolecular syntheses.[29-31] Even polymers that cannot be synthesized using conventional catalysts have been produced by *in vitro* enzymatic polymerization. For example, a cyclic monomer 2-dimethylaminotrimethylene carbonate (DMATC) could be polymerized enzymatically, while organometallic catalysts failed.[32]

Given the growing importance of environmentally friendly processes, alternatives are being sought to catalysts that require harsh reaction conditions. Furthermore, the use of synthetic materials for *in vivo* applications requires biocompatibility and the contamination of final products by catalysts is a common issue.[33-34] For example, N,N'-dicyclohexylcarbodiimide (DCC) used as a dehydrating agent in organic coupling and cyclization reactions and its hydrated form DCU (dicyclohexylurea) are highly toxic and difficult to remove completely from the final product. Transition metal catalysts are versatile

and powerful in metathesis chemistry, but the contamination of products by residual catalyst is hard to avoid.[34] Both are widely employed in the synthesis of polymerizable cyclic compounds, such as macrolactones, and enzymes can provide a non-toxic and biodegradable alternative to these compounds. [35-36]

The class of hydrolases (Enzyme Commission number EC 3) is the most utilized class of enzymes in the synthesis and modification of macromolecules, both in research and industrial manufacturing, owing to high stability, efficiency, commercial availability and broad substrate specificity.[37-38] Many industrial sectors such as detergent, leather, textiles, pulp and paper, food, dairy and biofuel industries depend on hydrolases. These enzymes traditionally catalyze reversible bond cleavage using water as a nucleophile, but function also in organic solvents, ionic liquids or supercritical fluids. Non-conventional reactions, such as C-C, C-X and X-X bond formation (X = heteroatom) and oxidative processes, that demonstrate the catalytic promiscuity of hydrolases have earlier been reviewed by Busto and coworkers.[39]

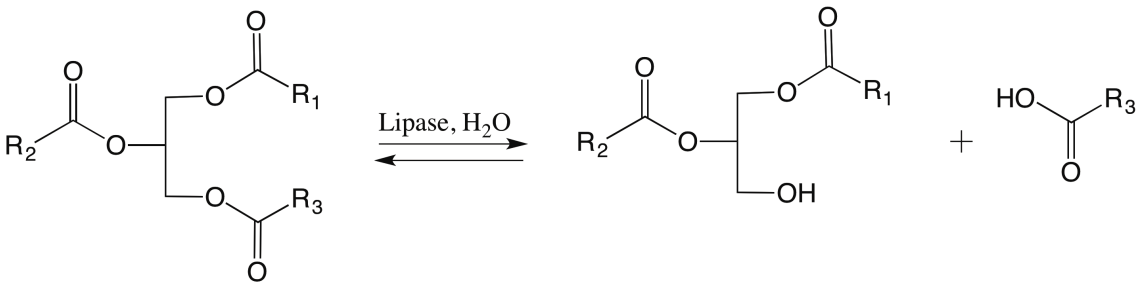
Among the hydrolases, proteases (EC 3.4) and lipases (EC 3.1.1.3) have gained most popularity in chemoenzymatic syntheses. While proteases have been described as the best understood class of enzymes in terms of structure-function relationships[40] and are powerful in the hydrolysis and synthesis of (poly)peptides or even polyesters,[39-42] the catalytic potential of lipases in hydrolytic, aminolytic, esterification and transesterification reactions is extensively documented[43-44] and during the last decades they have been introduced in a variety of chemo-, regio- and enantioselective polymerizations.[45-47] The selectivity and robustness of lipases allow for both the synthesis of cyclic monomers with functional moieties[45-46] and their enzymatic ring-opening polymerization (eROP). The functionalities provide a platform for

post-polymerization derivatization of materials and enable tailoring their mechanical properties, degradability and biocompatibility.[48-50] Recently, the versatility of lipases has been demonstrated in producing complex macromolecular architectures via chemoenzymatic methods.[51] In this review, we highlight the recent advances in the lipase-catalyzed synthesis of macrolactones and their polymers. Enzyme-catalyzed macrocyclization is particularly relevant as it can render complete synthetic pathways more sustainable. The selectivity of different lipases and factors affecting the reactivity will be presented as a primer to the latest developments and advantages of eROP techniques.

2.3 Lipases used in chemoenzymatic transformations

Lipases help in the digestion of lipids, more specifically in the hydrolysis of triglycerides acting at the organic-aqueous interface (Scheme 2.1).[52] The properties of lipases, such as specificity, thermostability and optimal pH, depend greatly on the source they are isolated from.[53] Their reactions are reversible depending on reaction conditions, making esterification, transesterification and hydrolytic reactions possible.[54] In fact, as soon as anhydrous conditions are created, ester bonds are formed instead of cleaved. However, other parameters, such as solvent polarity and by-product scavenging, can also affect the reaction balance. Lactonization and polymerization reactions are commonly catalyzed by microbial lipases from *Pseudomonas* species and *Candida antarctica* as well as animal lipase from porcine pancreas. These will be introduced below. The advantages of microbial lipases over the animal or plant ones are wide variety of catalytic activities, high yields, ease of genetic

manipulation, regular supply and rapid growth of microorganisms on inexpensive media. Microbial enzymes are also more stable and their production is more convenient and safer.[20]



Scheme 2.1 *In vivo* action of lipases in the hydrolysis of triglycerides.

2.3.1 *Pseudomonas cepacia* (PS)

PS lipase is known for its high enantioselectivity in asymmetric synthesis.[24] For example Ziarani *et al.* reported high yields and enantiomeric excess for a large array of syntheses, such as *S*-enantioselective esterification of racemic bromoalcohol present on heterocycles.[55] The catalytic activity of immobilized PS lipase in esterification reactions has recently been enhanced by sonication,[56] and combining high activity, reusability and very mild reaction conditions renders this emerging type of chemistry attractive for larger scale applications. In fact, the authors report a two-fold increase in efficiency in addition to using a more energetically sustainable technique. PS lipase from *Burkholderia cepacia* was also used by Matsumura and coworkers for the polymerization of a 6-membered lactide, which resulted in high conversions and molecular weights combined with low dispersity ($Đ_M$). In this case, PS lipase showed better potential than the four other lipases tested and gave the best results

when used at 130 °C.[57] Other enzymes such as *Candida antarctica* lipase B (CALB) are deactivated at such temperatures,[18] which makes one question the actual mechanism involved in this polymerization reaction. Indeed, at such high temperatures, cationic ROP is possible in the case where lactic acid impurities are present in the medium.

2.3.2 *Pseudomonas fluorescens* (PF)

PF lipase has been used especially in ring-opening polymerizations, and for instance Kobayashi group reported high conversions in the polymerization of δ-valerolactone using non-immobilized PF lipase.[27, 55] PF lipase also showed high activity in the copolymerization of lactones and divinyl esters and glycols, where both eROP and polycondensation took place simultaneously in one-pot reactions.[14, 58]

2.3.3 Porcine pancreatic lipase (PPL)

PPL is one of the most widely used lipases for chemoenzymatic transformations. One major advantage is its availability and ease of production.[20] In fact, PPL has been employed at industrial scale for racemic separations using a selective acylation principle. Zaks *et al.* reported that the dry version of PPL is thermally stable at 100 °C while demonstrating fast kinetics.[17] They also observed that in contrast to the wet counterpart, the dry enzyme does not catalyze reactions at bulky tertiary alcohol sites although this is possible by *Candida antarctica* lipase B (CALB).[59-60]

2.3.4 *Candida antarctica* lipase B

CALB possesses remarkable stability and catalytic efficiency, both in its free or more often immobilized form, for the formation of esters in organic media. CALB is a microbial lipase well appreciated because of the versatility of substrates it accepts.[61] It has been proven useful in the eROP of medium to large cyclic structures. Most lipases polymerize readily cyclic ε-caprolactone (ε-CL), and CALB has been used in eROP of ε-CL, both in bulk and ionic liquids.[62-65] For example, Deng and Gross obtained 80 % conversion in 4 hours in bulk at low amounts of immobilized enzyme.[66] Chemoenzymatic esterification has been used in the synthesis of adipic acid derivatives from four different isomers of butanol with the help of CALB. High yields were obtained, except in the case of the tertiary isomer.[59] The esterification and transesterification reactions on more bulky substrates, such as bile acids with large steroidal core, were also proven successful with this enzyme.[67] It has broad substrate specificity compared to other lipases, can tolerate temperatures up to 90 °C and is efficient in anhydrous organic environments. According to Uppenberg and coworkers, the crystal structure of CALB does not possess some of the common structural features of other lipases and in fact, these differences play a role in the substrate selectivity of CALB.[59, 68-69] Because of its wide use in macromolecular syntheses, we will mainly focus below on the use of immobilized CALB in lactonization and eROP reactions.

2.4 Substrate selectivity of CALB

2.4.1 Structure of CALB's active site

CALB, also commercialized under Novozyme 435, is the result of many years of research in protein engineering. In fact, its high versatility has led to various paths of optimization and some of them were highlighted in previous reviews.[61, 70] Structural modifications play an important role in enzyme activity, and methods are being developed to facilitate their engineering. As an example, the assembly of designed oligonucleotides (ADO) of Lipases A and B resulted in a two-fold improvement in conversion and enantioselectivity, compared to previously used methods.[71]

As a result of synthetic enzyme evolution, the binding pocket of CALB can fully accommodate linear substrates with up to 13 atoms in its elliptical, steep funnel with dimensions of 9.5 x 4.5 Å (Figure 2.1) and a lid constituting the higher part of the wall (10.5 Å).[15, 69] The serine moiety, part of the active amino acid triad, is located at the bottom of the funnel. CALB is particularly interesting, because it possesses amino acid motifs that are not present in most hydrolases, and because it possesses an unusual amount of polar side chains near its active site. The narrow shape of the funnel allows strong stereospecificity on chiral substrates during hydrolysis and esterification reactions.[15]

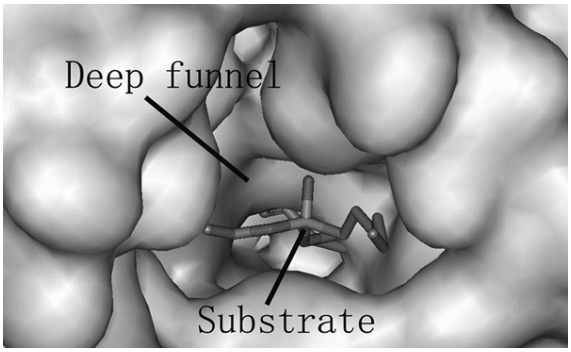


Figure 2.1 Deep funnel-shaped binding pocket of CALB with active site buried inside. Reproduced with permission.[52] Copyright © 2010 by the American Society for Biochemistry and Molecular Biology.

2.4.2 Esterification and lactonization

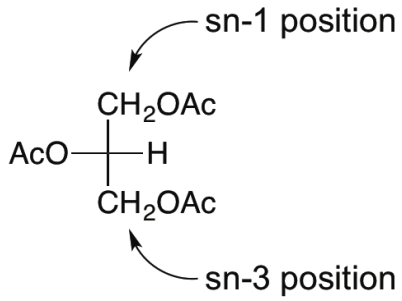
The length of the alkyl spacer in hydroxy acids or esters is of critical importance in enzymatic intramolecular lactonization reactions, because not all enzymes possess the same shape of binding pockets.[15] The relationship between the alkyl chain length and the expected yield of esterification or hydrolysis follows a different model for each enzyme, which makes these reactions different from conventional chemical syntheses. The optimal length of fatty acids for such reactions using different kinds of lipases have been investigated.[52] Pleiss *et al.* stated that CALB demonstrates high selectivity towards small and medium chain-length fatty acids, but that the selectivity decreased with increasing length of fatty acid.[15] Kirk *et al.* showed somewhat different results on the effect of aliphatic carboxylic acid chain length for esterification reactions with glucose using CALB. Almost identical conversions were reached for acids containing 10 to 18 carbons, with CALB having a preference for hexadecanoic acid (C_{16}) over octanoic (C_8) and hexanoic acids (C_6). However, a pronounced competitive effect

was observed when 9-*cis*-octadecenoic acid (C_{18}) was used together with hexadecanoic acid, showing that compounds with double bonds are not favoured, probably because of the narrow structure of the active site of CALB.[72] Rangheard *et al.* also performed such alkyl length competition experiments for lactonization on other enzymes than CALB.[73]

While the chemical lactonization reactions of hydroxyalkanoic acids tends to depend solely on ring strain and alkyl chain rigidity, bioorganic catalysis is more size-dependent and stereoselective. Kageyama *et al.* obtained high yields of cyclization (52-65 %) using lipase *Pseudomonas sp*. for substrates with aliphatic chain length of 17-18 carbons,[74] while shorter and longer chain lengths led to less cyclization. The highest conversion to diolides was obtained at a chain length of 10. Furthermore, CALB was shown to be stereospecific towards *R*- or *S*-enantiomers depending on the ring size.[75] Indeed, pure enantiomers of 6-methyl-**ε**-caprolactone were obtained with the use of CALB.[76] The *S*-enantiomer was selectively hydrolyzed, separated from the *R*-enantiomer, and lactonized using the same enzyme.

Lipases possess an sn-1 and/or sn-3 specificity towards triglycerides, depending on their structure. This corresponds to a preference towards primary alcohols (Scheme 2.2).[77] For CALB, Mohd *et al.* concluded that the maximum yield of esterification is independent of the alcohol structure for primary and secondary alcohols, since they obtained yields > 96 % for different primary and secondary isomers of butanol.[59] However, these alcohols required different reaction conditions. As for tert-butanol, the reaction rate and yield were significantly lower (39 %). This is also what Kourist and Bornscheuer observed with most commercial enzymes.[60] Some lipases other than CALB contain a specific amino acid motif with glycine and alanine groups in their oxyanion binding pockets and are active towards tertiary alcohols.[59-60, 78] One of the well-known lipases in this category is *Candida rugosa* lipase

(CRL). According to the protein data bank, it possesses an alcohol-binding pocket that is 1.5-2 Å wider than with most lipases that do not accommodate tertiary alcohols. The characteristic glycine amino acid sequence is also found in the active center of CRL.[15, 77]



Scheme 2.2 Reactive centers of triglycerides towards lipases.

2.4.3 Enzymatic ring-opening polymerization

Similar principles for substrate selectivity apply to eROP, where the active site of the enzyme must accommodate the monomer in a correct position. In some cases, it is possible to design the substrate or select the enzyme as a function of the size and structure of the substrate using the transition state analog substrate (TSAS) principle.[27, 79] TSAS consists of using molecular modeling to select a right pair of enzyme and substrate for efficient conversion. It is based on a principle that a monomer possessing shape and structure similar to the transition state of the active site of an enzyme will more readily bind to it. This selectivity design was established from the fact that when the enzyme is moulded to best fit the transition state substrate, the energy barrier decreases. As an example, molecular docking studies (Figure 2.2) performed on cholic acid (CA), a bile acid bearing a carboxylic acid moiety and three hydroxyl groups in a bent steroid ring, revealed that the acid group on position 24 (Scheme 2.3) readily reacts when

exposed to CALB. However, only once this position is esterified and becomes less polar, CA positions itself in a favourable way for the hydroxy group on position 3 to react.[68] This is one of the reasons why enzymatic condensation polymerization would not work using CA as the monomer. The hydroxy groups at positions 7 and 12 are too far away from the active triad because of the lack of flexibility of the steroidal structure (Fig. 2.3).

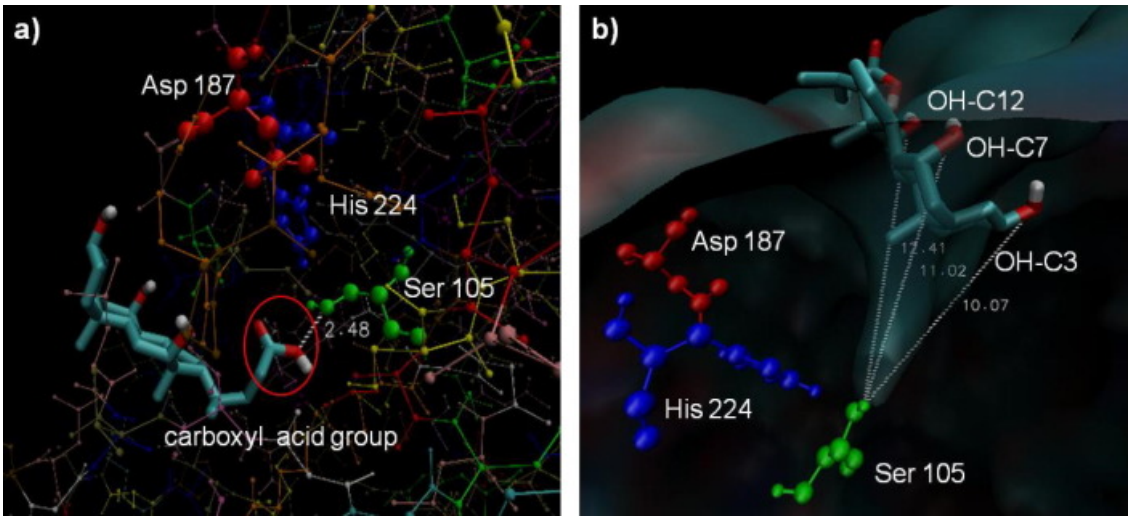
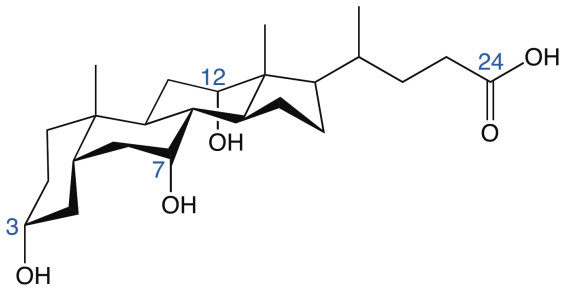


Figure 2.2 Docking of CALB with CA: interaction of substrate with Ser 105 of active site trough: (a) the carboxyl moiety; (b) hydroxyl groups. Reproduced with permission.[68] Copyright © 2016, Elsevier.



Scheme 2.3 Structure of CA with labeled reactive centers.

The effect of ring size on the outcome of the eROP using CALB was also investigated.[80] Using molecular dynamics and docking studies, Veld *et al.* determined that cisoid type lactones, such as δ-valerolactone (δ-VL) and ε-CL, were much less reactive in enzymatic polymerization reactions than larger transoid rings (Scheme 2.4), such as heptanolactone, dodecanolactone and pentadecanolactone. Van der Mee *et al.* obtained similar results with kinetic evaluation.[81] These results are different from the lactone reactivities in conventional ROP, which follows the concept of ring strain, but they could explain trends in enzymatic polymerizations that were not previously explained. The ring size was also shown to have an influence on the selectivity of *R*- and *S*-enantiomers for eROP.[75] As described above, it is the cisoid vs. transoid conformation that determines the outcome, as rationalized by van Buijtenen *et al.* Overall, CALB polymerizes the smaller lactones with ring sizes ≤ 7 atoms with a moderate selectivity towards the *S*-enantiomers of methylated lactone, while the larger lactones with ring sizes ≥ 8 exhibit are polymerized with a pronounced preference towards the *R*-enantiomers of the same species.[75] Peeters et al. took advantage of this high stereospecificity to successfully obtain chiral block copolymers.[82] This circumvents the need to buy enantiomerically pure monomers when enantiopure polyesters are desired.

Scheme 2.4 Transoid vs. cisoid conformation of the ester bond in lactones.

2.5 Immobilization of lipases and effect on reactivity

CALB is typically provided in its immobilized form, as immobilization facilitates the reuse of the enzyme and purification of products.[83] For example, commercially available Novozyme 435 is composed of spherical acrylic resin beads onto which the lipase is adsorbed. One way to quantify the performance of immobilized lipases is to use propyl laurate units (PLU) obtained per gram of product after performing a synthetic activity assay. This assay consists of measuring the amount of enzyme needed to obtain 1 μmol of propyl laurate per minute at 60 °C .[78] Novozyme 435 typically possesses an activity of 7000-10 000 PLU/g.

A distinguishing property of lipases in contrast to esterases is interfacial activation. When exposed to a water-oil interface, the secondary structure, often called the 'lid', exposes the active site. In strictly hydrophilic conditions, the lid remains in closed conformation, [52] and this is one reason why hydrophobic organic solvents are often preferred for chemoenzymatic synthesis.[84] Enzymes possess variable sizes of lids, depending on their nature. It was however shown that some lipases, such as CALB and *Pseudomonas glumae* lipase, possess an amphiphilic lid that does not show interfacial activation.[85]

Luckily, the non-covalent immobilization not only allows the reuse of CALB but also enhances its activity 7-fold without the need of additional genetic engineering. In fact, although the crystal structure determination of Novozyme 435 revealed an open conformation of the lid, the access to the active Ser-His-Asp triad site remains rather restricted, since interfacial activation does not take place when the enzyme is free of a solid support. [69, 86] Hence, the change in catalytic activity in relation with the immobilization was explained by Zisis *et al.* in 2015 upon theoretical and experimental modeling studies. The spherical

substrates were found to expose the active site of the enzyme, amplifying the activity towards bulky substrates, such as bile acids.[16, 87]

Since CALB is usually adsorbed instead of covalently immobilized on inert substrates, precaution has to be taken to avoid rapid leaching of the catalyst. In fact, although this is not a problem for biocompatibility, problems such as early degradation of the final product and lower efficiency in the reuse of the enzyme are possible.[16, 86] In fact, the resin beads are often brittle and are easily crushed upon magnetic stirring. This is why researchers often use shaking incubators when the reuse of the catalyst is desired. Although previous studies have shown good reusability of Novozyme 435 of up to 10 cycles,[88-89] some obtained different results,[90] probably because of differing methods of agitation. One novel way to avoid leaching is to coat the immobilized enzyme with ionic liquids. He *et al.* were able to coat the acrylic resin beads with different alkyl chain lengths.[91] Not only did it prevent leaching but this coating also improved the access of the substrates to the active site. Similar results were obtained in 2012 by Dong *et al.* when they coated Novozyme 435 with ionic liquid 1-butyl-3-methylimidazolium hexafluorophosphate ([BMIM][PF_6]) to polymerize 1,4-dioxan-2-one (PDO).[92] The molecular weights were much higher when the enzyme was coated than when using [BMIM][PF_6] as a solvent (Figure 2.3).

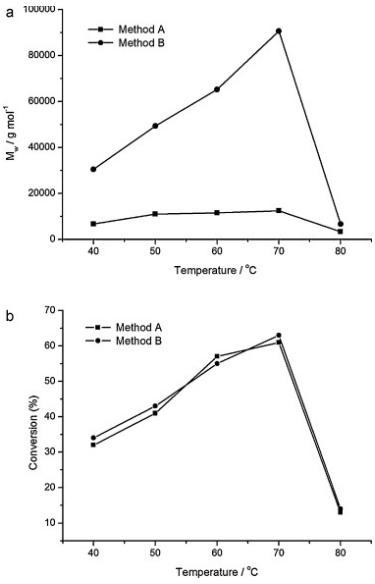


Figure 2.3 Polymerization of 1,4-dioxan-2-one at different temperatures A) using ionic liquids as the solvent and B) using ionic liquids for coating Novozym 435. Reproduced with permission.[92] Copyright © 2012, Elsevier.

Efforts have previously been made in covalent linking of lipases to their solid supports, but adsorbed enzymes are still preferred. In fact, although covalent linking allows better thermal stability and reusability, adsorption allows more enzyme units per surface area, which diminishes the amount needed.[91] Furthermore, the covalently bonded enzyme may restrict the access of substrates to the active site of enzyme moieties, or even change the nature of the active site.[83, 93] Comprehensive reviews on immobilization were previously published.[83, 94]

2.6 Lactonization and ring-opening polymerization

2.6.1 Esterification vs. transesterification and lactone formation

Both esterification and transesterification reactions are possible using lipases, which often show different catalytic potentials for these reactions.[95] Kotogan *et al.* showed that the

transesterification activities of two types of lipases were up to 62 times higher than their esterification activity in 20 M n-heptane.[96] The esterification reactions still occurred, but the interactions between substrates and lipases were less specific and much slower. Similar results were obtained by Pirozzi *et al.* with CALB, which revealed reduced stability in the presence of acidic reagents and released water, especially in solvent-free media.[97] They emphasized that 45 and 60 °C are the optimal temperatures for esterification and transesterification, respectively. The impact of released water is more important in solvent-free media than under high-dilution conditions where lactones are desired.

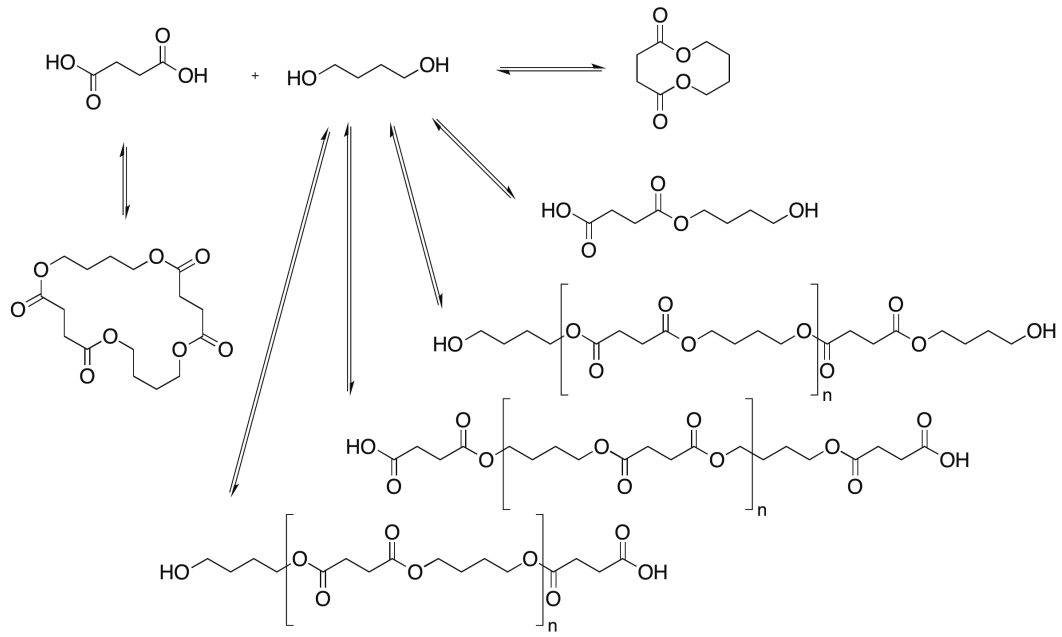
Transesterification reactions also favour the easy removal of by-products, such as methanol. When by-products are removed during the reaction, the reaction equilibrium shifts towards the products. One way of doing so while maintaining inert conditions is to use Dean-Stark apparatus. Another cause for the ease of transesterification reactions is the fact that some esters, such as vinyl esters are better leaving groups, because they form keto-enol tautomers.

Considering that esterification and transesterification reactions are achievable using lipases, it is not surprising that they could also accommodate lactone formation via intra- and intermolecular reactions. A major difference between the chemical and biochemical lactonizations is the diversity of final products. While chemical catalysis often results in the production of the lactone and side-products, such as linear oligomers, lipases will also favour the cyclization of these linear oligomers, yielding diolides, triolides, and so on.[98] In fact, in the case of CALB, molecular docking studies clearly demonstrated the compatibility with bulky substrates, such as cholic acid, and hence, macrolides with bulky substrates can also be synthesized.[68, 80] Nevertheless, reaction conditions should be optimized to ensure the highest yields possible. Parameters such as the size of substrate, dilution, type of lipase, presence or

absence of immobilization, polarity of the solvent and temperature need to be considered to obtain the right product in high yields, as discussed in the upcoming sections.[99] As an example, high dilutions usually result in higher yields for lactone formation, but lower yields for polymerization reactions.

2.6.2 Effect of concentration

To make sure that oligomers and polymers are not the main products when lactones are desired, the reaction conditions are adjusted according to the Jacobson-Stockmayer theory, which defines the principles of ring-chain equilibrium.[100] An important parameter in intramolecular cyclization is the effective molarity (EM), which defines the probability of two ends of a chain finding one another. To obtain predominantly cyclic products, the initial substrate concentration should be much lower than the EM[101] and therefore, high dilution is usually required. This is true for both chemical and enzymatic cyclization reactions, both considered to be of first-order kinetics.[102-103] Other options to push the equilibrium towards cyclization involve distilling the cyclic structures as the reaction proceeds and evaporating the alcohol or water produced from transesterification.[104] A multitude of sizes of cyclic ester oligomers and linear ester oligomers are to be expected when reacting a diacid and a diol, as described by Habeych *et al.* and shown in Scheme 2.5.[98]



Scheme 2.5 Products from the polycondensation of succinic acid and 1,4-butanediol catalyzed by CALB.

Concentration affects directly the reaction rate and ring-chain equilibrium shifts towards a higher yield of cyclic products under dilute conditions, unless the macrocyclic structures are removed along the way.[38, 98] Most publications have demonstrated this shift in equilibrium at concentration range of 0.001 - 0.02 M.[49, 74, 105] Shen *et al.* concluded that according to the Michaelis-Menten equation of enzyme kinetics, which relates the concentration of the substrate to reaction rate, an excessive amount of substrate would produce undesired products. Although increasing the concentration of the substrate increases the reaction rate, an excessive amount could result in two of more substrate molecules entering the same active site of lipase. This increases the probability of substrate molecules combining to form unwanted products, such as oligomers.[106]

2.6.3 Substrate molar ratio

To avoid unnecessary hydrolysis when performing intermolecular lipase-catalyzed esterification reactions, there should be excess acyl donor molecules in the reaction media.[107-108] Occupying the enzyme free active sites once they have catalyzed esterification prevents the hydrolysis of already formed products.[109] In the case of lactonization reactions, an equimolar ratio is preferable to push the equilibrium towards cyclization, as an excess of acyl donor could result in larger extent of oligomerization.

Following the ring-chain equilibrium-principle, high concentrations are required to achieve high molecular weights in polymerization reactions.[98-99, 110] Polymerization is preferably performed in bulk when possible and the first successful eROP using lipases was indeed performed in bulk.[111] In such concentrated conditions, great care has to be taken to make sure the reaction medium stays practically anhydrous. Although traces of water may help in the initiation step and result in polymers belonging to the class of telechelics, too much of it will induce hydrolysis, resulting in high $Đ_M$ and low molecular weights. In fact, Strandman *et al.* stated that the cost to obtaining high molecular weights is having a more moderate conversion.[13] Therefore, the conditions need to be adjusted according to desired product.

2.6.4 Drying of reagents

For all chemoenzymatic esterification reactions, it is of common practice to dry the reagents and enzymes prior to synthesis to allow a maximal conversion. This is particularly important in lipase-catalyzed reactions, as the natural function of lipases is to hydrolyze ester bonds in the presence of water. Gorke *et al.* reported a significant activity difference and

increase in molecular weight (from 6300 to 9700 g/mol) when immobilized CALB was dried in a desiccator prior to polymerization.[112] de Geus et al. also extensively studied drying methods and obtained the highest monomer conversion (95 %) when the enzyme was dried in a Schlenk flask containing activated molecular sieves, in a desiccator containing P_2O_5 for 16 h at 50 °C under vacuum.[113]

Knowing that enzymes require a minimal layer of water at their interface to function, one might wonder whether drying the enzymes prior to reaction really is beneficial. Some state that drying the enzyme removes the essential water layer found on the surface, altering the catalytic efficiency (see solvent choice section). However, Badgujar *et al.* showed very recently that the essential water contained on the surface of the enzyme was so tightly bound that it is stable until about 250 °C.[56] This test was performed to determine the stability of the water layer, knowing that the enzyme's activity decreases at temperatures over 80 °C.[114] Hence, drying Novozyme 435 over P_2O_5 under vacuum at mild temperatures preserves the activity of the lipase, while removing the excess water.[112]

2.6.5 Removal of by-products

Esterification and transesterification reactions depend on an equilibrium that is often governed by the amount of water or small esters present in the reaction medium. Several research groups report the use of molecular sieves to further push the equilibrium.[98, 105, 115-116] Duan *et al.* used CALB for the esterification of rutin, and found that the addition of 100 g of 4 Å molecular sieves per liter of solvent after 24 h reaction time increased the yield from about 15 % to 46 %.[115] Figure 2.4 shows the influence of molecular sieves on the conversion in the

esterification of rutin. The sieves were added in the beginning of reaction, as the time of the addition of drying agent has an influence in the final conversion.

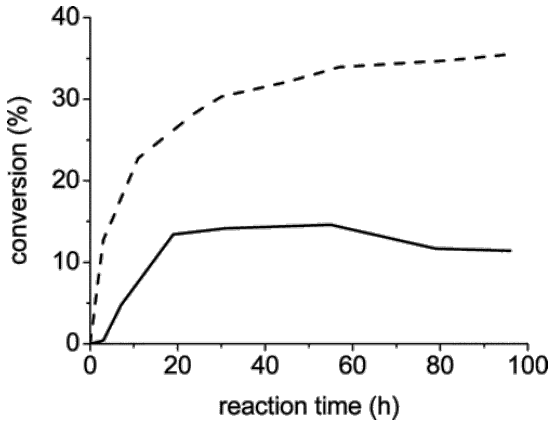


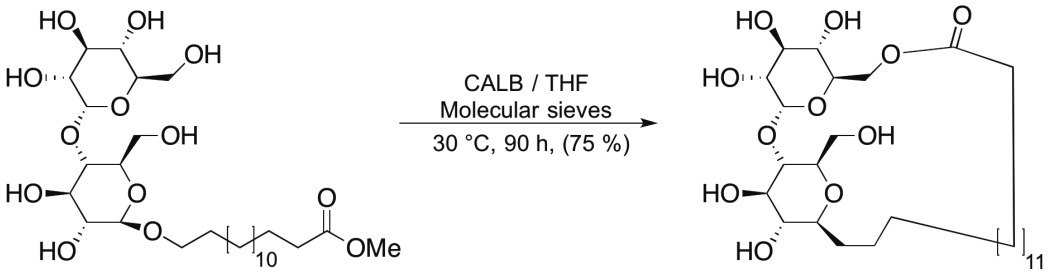
Figure 2.4 Time dependence of the conversion of rutin in lipase-catalyzed esterification in the presence (dashed) or absence (full) of molecular sieves. Reproduced with permission.[115] Copyright © 2006, American Chemical Society.

Adding drying agents may seem like a simple and efficient solution to scavenge by-products, but there are some drawbacks. Vigorous mechanical stirring crushes the zeolite beads,[89] leading to increased viscosity of the solution and even more diluted conditions will be needed. Matsumura *et al.* came up with the idea of using zeolites suspended in the vapour phase of the reaction flasks.[117] They described their dehydration trap as a Dean-Stark apparatus packed with molecular sieves and took advantage of the azeotropic nature of toluene-water mixture in a polymerization reaction.[118] Ducret *et al.* also took advantage of molecular sieves in a Soxhlet apparatus and applied reduced pressure to continuously condense the wet solvent, which inevitably passed through a patch of dried sieves.[119] In contrast to condensation polymerization, eROP simplifies the course of the reaction, because

instead of producing water or alcohol molecules, it uses the traces already present in the reaction to initiate polymerization.[14] As water is not released, there is no need for by-product scavenging techniques in eROP.

2.6.6 Temperature

Although enzymatic esterification reactions are possible using CALB under very mild conditions (< 45 °C), lactonization reactions often require temperatures in the range of 55-75 °C. Sih *et al.* reported an excess oligomeric products at temperatures ≤ 45 °C, when using *Candida cylindracea* and *Pseudomonas sp*. lipases and PPL.[49] Cyclization reactions generally possess a higher energy barrier. However, the more versatile CALB does not always require high temperatures and very high dilutions to catalyze esterification reactions. For example, Bisht *et al.* obtained excellent yields of macrolides of about 75 % when performing the ring closure of glycolipid analogs at 30 °C using CALB (Scheme 2.6).[116] The particularly good yields were explained by these types compounds being synthetic analogs of a microbially produced lactones.[120] Fortunati and coworkers observed that 45 °C was an optimal temperature in the synthesis of musky macrolactones.[48]



Scheme 2.6 Synthesis of glycolipid macrocycles.

The temperature used for eROP has a clear impact on the molecular weights of polymers and their degrees of crystallinity. In general, temperatures of 60-80 °C result in high catalytic activity of lipases in most low-polarity solvents.[13] For example, Ozsagiroglu *et al.* obtained higher conversions in shorter times at 60 and 80 °C than at 40 °C in the synthesis of polycaprolactone (PCL) in n-hexane and diisopropyl ether. In toluene, the highest molecular weights were obtained at 40 °C,[121] which is in accordance with the findings of Yang *et al.* where 45 °C reaction temperature yielded higher molecular weights than 60 °C. Increasing reaction time from 24 to 48 h led to a considerable decrease in molecular weight, probably due to more predominant hydrolysis and transesterification reactions after all cyclic monomers were consumed.[122] When PEG was used as a macroinitiator in the eROP of ε-CL in toluene, the temperature of 80 °C yielded significantly higher conversions and molecular weights than 60 °C. Lower temperatures were not tested in this study.[123] Recently, Wilberth *et al.* showed that increasing the polymerization temperature from 70 to 90 °C changed the properties of poly(pentadecanolide) (PPDL) significantly. The average molecular weight and crystallinity were lower when the reaction was performed at 90 °C, while the $Đ_M$ was relatively high in both cases. The lower crystallinity arose from higher number of terminal OH groups that disrupt the crystallization.[124]

2.6.7 Amount of enzyme

High amount of enzyme or rather high activity in PLU units leads to faster reaction up to a certain threshold value after which the rate remains stable.[125-126] Since the amount of enzyme is directly related to the amount of active sites for acyl-enzyme complexation, a clear

linear increase in the yield at constant time has been observed.[59] An extrapolation of the data indicates that the rate is affected, but not necessarily the final yield. Very often, the same weight will be used for both initial substrates and immobilized CALB.[105] For example, Matsumura *et al.* found that a maximum yield was obtained at 100 wt-% of CALB to monomer (203 g/mol). The yield did not increase with higher amount of enzyme. Other researchers such as Ducret *et al.* have used similar amounts of enzyme per mole of substrate (180 g/mol).[119] Some research groups have used a very high amount of enzyme (2000 g/mol) for their synthesis at comparable reaction times.[67]

Lipase-catalyzed eROP requires lower amount of enzyme than lactonization, because the enzyme has a direct influence on the number of growing chains, that is, degree of polymerization (DP_n) and $Đ_M$. The amounts of immobilized CALB in eROP reactions have been 0.44 - 10 wt%. [13, 66, 107] Nevertheless, for the same reaction time, the conversion increases when more catalyst is used. For example, Sobczak *et al.* reported conversions of 49, 82 and 85 % after 14 days using 1.25, 2.5 and 5 wt % CALB in PEG-initiated polymerization of ε-CL.[123]

2.6.8 Solvent

As discussed above, the polarity of solvent affects the activity of lipase. When using the logarithm of octanol-water partition coefficient (log P) as a quantitative measure of solvent polarity, higher log P, that is, lower polarity of the solvent, is associated with better activity of lipase. Laane *et al.*[127] have attempted to establish rules to optimize biocatalysis and concluded that solvents with log P > 4 are suitable for bioorganic synthesis, solvents possessing a log P between 2 and 4 are rather unpredictable, and all solvents with a log P < 2 distort the essential water layer. Kumar and Gross determined similar correlations for eROP using CALB, shown in Figure 2.5.[18]

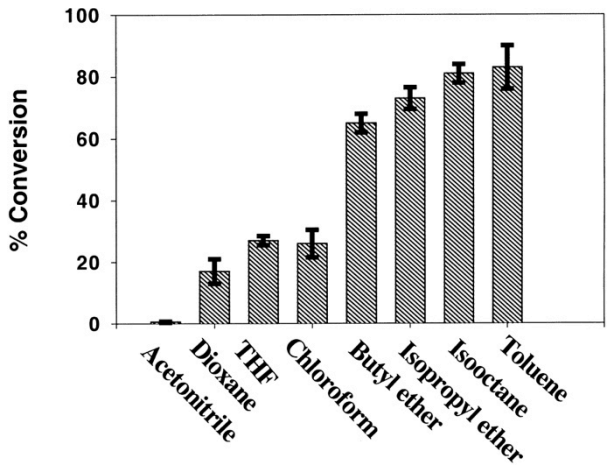


Figure 2.5 The effect of solvent on the conversion of CALB-catalyzed polymerizations of ε-CL. Reproduced with permission.[18] Copyright © 2000, American Chemical Society.

It has been suggested that hydrophilic solvents of reaction media may remove or 'dissolve' the water layer vital to the enzyme.[24, 106] Zaks *et al.* stated that depending on the origin of the lipase, the water molecules would be more or less tightly bound to the enzyme and that the water layer could instead be simply distorted or displaced.[127-128] For example, pancreatic lipases are particularly stable in a variety of solvents as the water is tightly bound to the enzyme and many lipases can still maintain robust properties in a 99.98 % water-free environment. After performing molecular dynamics simulations, Li *et al.* proposed a more logical explanation to the destabilisation of the lipases in polar solvents. They demonstrated that polar solvents can enter the active site, interact strongly with it and disrupt the hydrogen bonding the Ser^{105} and His^{224} amino acids (Figure 2.2).[52] This phenomenon was not observed for non-polar solvents.

In addition to polarity, the molecular structure of the solvent may play an important role. Sasi *et al.* reported different results for cyclic and linear solvent molecules and

cyclohexane resulted in better yields than linear n-hexanes, even though the latter possesses higher log P value.[52, 128-129] Similar results were obtained by Yang *et al.* for the polymerization of ε-CL.[122] Lactonizations showed higher yields in isooctane than in octane although their log P values are nearly the same.[129] It also seems that polymerization of ε-CL using CALB works particularly well in toluene, probably for solubility reasons, as higher molecular weights were obtained in toluene than in n-hexane despite the lower log P of toluene.[121, 130]

2.7 Recent eROP techniques and architectures using CALB

For several types of monomers, condensation polymerization is not possible or it results in low molecular weight products, because the method is limited by the removal of the condensate. This often requires the use of vacuum or high temperatures, which is not ideal for thermally unstable monomers and oligomers.[122] This is the case with polybutylene succinate (PBS), which almost tripled in molecular weight when an eROP strategy was employed.[131]

In the following section, novel reaction conditions and architectures of polymers using lipases will be outlined, showing the progress made since 2006. Several reviews have been published about polymerization reactions involving lipases,[14, 28, 132-133] but our focus is on the work done within the last ten years.

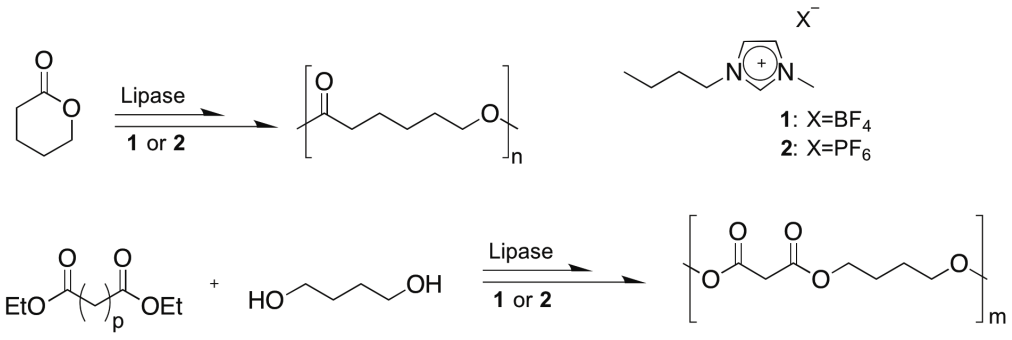
2.7.1 Methods for more sustainable synthesis

2.7.1.1 Ionic liquids as reaction media

The combination of enzymatic catalysis and environmentally friendly media such as ionic liquids represents a new type of green catalysis. Ionic liquids are salts with melting

points below 100 °C and are interesting as solvents owing to their very low vapour pressure and their high thermal stability.[64] Several research groups have reported enhanced thermal and functional stability of CALB in ionic liquids,[134-135] while Kim et al. showed that the enzyme's conformation change in ionic liquids is not necessarily beneficial, depending on the ionic liquid used.[136] In addition, 1-alkyl-3-methylimidazolium-based ionic liquids offer a reaction medium that accommodates more polar substrates while maintaining the enzyme activity, in contrast to polar organic solvents.[137]

Ionic liquids were first employed in enzymatic polymerization reactions by Uyuma *et al.* in 2002, using CALB (Scheme 2.7).[64, 138] High conversions of up to 97 % were obtained when performing eROP of ε-CL. Ionic liquids also allowed high conversions for polycondensation reactions involving dicarboxylic acids with diols, as the very low volatility of ionic liquids allowed the use of reduced pressure to remove the ethanol produced upon the reaction. The conversion increased from 50 to 82 % and number-average molecular weight M_n increased from 350 to 1100 g/mol, when the pressure was reduced from 760 to 60 mmHg.



Scheme 2.7 eROP of ε-CL and polycondensation of dicarboxylic diesters with 1,4-butanediol in 1-butyl-3-methylimidazolinium tetrafluoroborate (**1**, [BMIM][BF_4]) and in butylmethylimidazolinium hexafluorophosphate (**2**, [BMIM][PF_6]).[64]

The molecular weight of PCL synthesized in ionic liquids was further improved by Wu *et al.* when they used dicationic ionic liquids. In fact, they are known to be more viscous and dense than their monocationic counterpart, which is beneficial to maintain the activity and stability of enzymes for longer periods.[139] Maximum conversion and M_n were 62 % and 26,200 g/mol, respectively, obtained upon adjusting parameters such as lipase/ε-CL/ionic liquid ratio, alkyl chain length, reaction time and temperature in different monocationic and dicationic media.[139] Gorke *et al.* investigated eROP reactions of 4-7-membered lactones in ionic liquids and synthesized poly(hydroxyalkanoates) (PHAs) in [BMIM][Tf_2N], which was the best solvent for these polymers. The highest molecular weights using immobilized CALB were obtained with β-propiolactone and ε-CL, 11900 and 9700 g/mol, respectively.[112]

Gumel and coworkers investigated the use of ionic liquids together with ultrasonic irradiation. They compared polymers obtained with and without the use of sonication and found that sonication enhanced the molecular weight, conversion and kinetics of the eROP of ε-CL. In fact, although the polymerization mechanism remains the same, the ultrasound irradiation increased the average length of the polymer and considerably reduced the $Đ_M$.[140]

2.7.1.2 Aqueous dispersions

In an attempt to render reactions even greener, aqueous reaction media have been explored. Remembering that lipases' natural function is the hydrolysis of ester bonds in such media, this presents a substantial challenge. Nallani et al. attempted to polymerize several cyclic monomers in aqueous media in polymersome nanoreactors in 2007, but determined that only the more hydrophobic monomers could do so. In fact, 8-octanolactone and 12-

dodecanolactone were successfully polymerized with M_w of up to 2701 g/mol, while the more polar ε-CL and 4,7,10-tetraoxacyclotetradecane-11,14-dione did not produce any polymer.[141] In 2014 however, ε-CL was successfully oligomerized in a one-pot reaction using aqueous dispersions combined with CALB catalysis.[142] As expected and confirmed by SEC, the polymer chains were hydrolyzed as the reaction went on, resulting in a higher proportion of oligomers. Oligomers (number-average $DP_n = 8$) were obtained with 72 % yield and polymers (number-average $DP_n = 38$) were obtained with 26 % conversion when immobilized CALB was employed. Other forms and types of lipases were tested, but they did not give conclusive results.

2.7.1.3 Microwave radiation (MWR)

Although MWR-assisted enzymatic synthesis has been mildly explored for small molecule transformations, very few investigations on the polymerization counterpart exist. In 2006, Kerep and Ritter showed that in some solvents, the thermal energy to obtain reflux could be replaced by microwave radiation for the eROP of ε-CL.[143] The polymerization of the same monomer was also investigated in bulk conditions.[144]

2.7.1.4 Recycling of polymers

Matsumura *et al.* discovered a way to recycle biodegradable polycarbonates in 2001 by taking advantage of the reversible hydrolytic nature of lipases.[145] Polymers were hydrolyzed into linear and cyclic oligomers in toluene containing water, as predicted by ring-chain equilibrium. To regenerate the polymers, this mixture was then placed in a slightly reduced pressure environment and water was removed azeotropically. As a result, higher

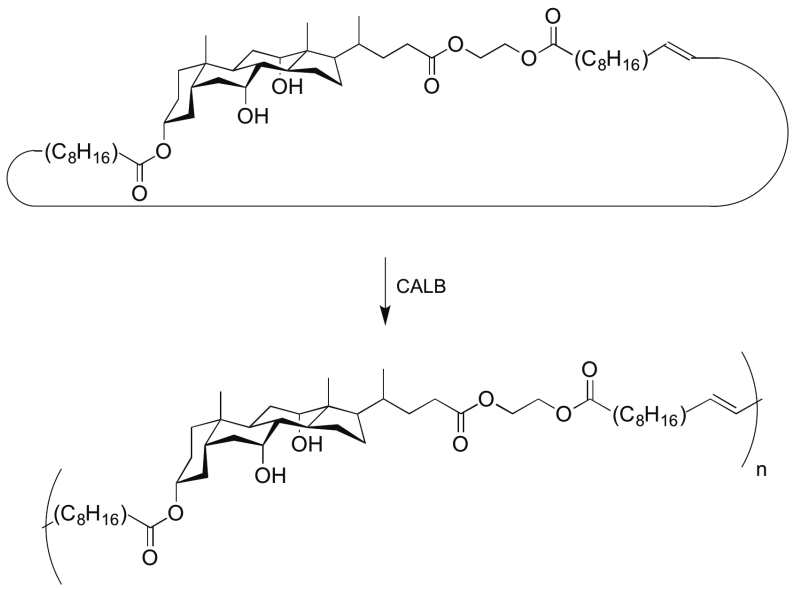
molecular weights were obtained than with eROP polymer (79 000 vs. 25 000 g/mol). More recently, the same group found a way to recycle cross-linkable polyesters using CALB. In fact, depolymerisation exclusively yielded cyclic oligomers, suggesting that identical yields could be reached for a second polymerization.[146] Yagihara and Matsumura also demonstrated the recyclability of thermoplastic elastomers after using CALB for the copolymerization of diols and succinate monomers.[147] They recovered identical molecular weights after recycling. In recent years, other research groups have demonstrated the enzymatic recyclability of polymers by reactive extrusion[148] and ring-chain equilibria.[149]

2.7.2 Polymerization of bulky substrates

The need to adjust the material properties and functionalities has driven the researchers to explore the use of lipases in the polymerization of larger and bulkier macrocycles. Bile acids are large steroidal structures that are naturally derived and possess several hydroxy sites for functionalization, as shown in Scheme 2.3 for cholic acid (CA). Bile acid-based polymers have shown potential for the tuning of thermo-mechanical properties, as well as thermal shape memory.[9, 12-13, 38] In 2010, Manzini *et al.* performed enzyme-catalyzed polymerization reactions involving macrocycles of various sizes, with and without large steroidal structures.[150] Although most reactions were successful, attempts at polymerizing dimers and trimers of lithocholic acid (LCA) gave no polymer. The authors assumed that this was caused by the lack of reactivity of secondary ester bonds. They later on hypothesized that the alpha face of LCA bearing functional hydrophilic groups is not accessible to the active site of the enzyme, since it is turned inward in the macrocycle. However, the reduced reactivity could also arise from the large size and lack of flexibility of such cyclic molecules. As

mentioned earlier in this review, the cavity of CALB leading to the active site is rather narrow and funnel-like.[15] The fact that the LCA molecules with the same secondary ester bonds were polymerized with satisfactory molecular weights when flexible alkyl spacers were added between the moieties supports both theories.

Cholic acid-based polymers have been synthesized using immobilized CALB, yielding molecular weights of 30 000 g/mol and low $Đ_M$ of about 1.3 (Scheme 2.8).[13] Although conventional ROP using transition metal catalysts yielded polymers with higher MWs and probably different head-to-tail and tail-to-tail arrangements, the shape memory and thermo-mechanical properties of enzymatically produced polymers with lower molecular weights were comparable.[15, 150] It was further suggested that the eROP of such macrocycles are not entropy driven (ED), as Manzini *et al.* described, but rather occur via transesterification. Indeed, the typical molecular weights obtained in lipase-catalyzed polymerizations were much lower than in entropy-driven ring-opening metathesis polymerization (ED-ROMP).[150]



Scheme 2.8 eROP of CA-based macrocycle.

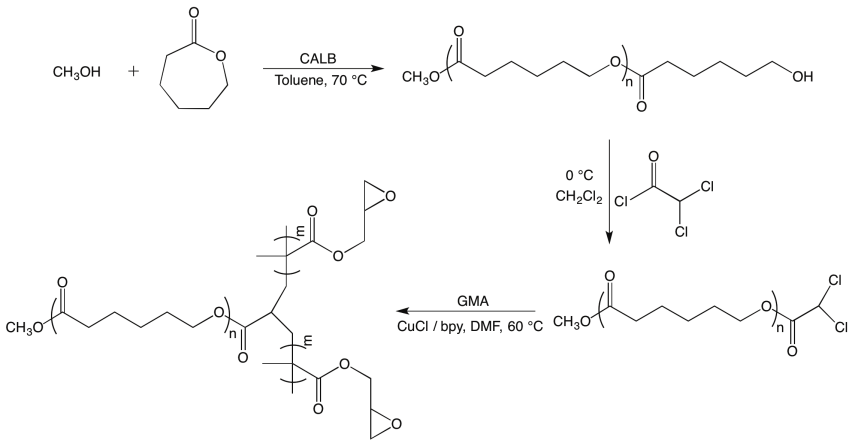
Hodge *et al.* further investigated the eROP of different architectures of bile acid-containing macrocycles.[151] Their study revealed, as Zigolo *et al.* also determined in 2016 by molecular docking studies,[68] that the hydroxy groups at positions 7 and 12 do not react when lipases are employed for catalysis. Indeed, polymerization did not occur when an alkyl spacer was linked to this position. They also ascertained the order of reactivity for the ester bonds (identified in Scheme 2.3) on bile acid-containing macrocycles (3α-OAc > 26 OAc >> 24-CO_2R), which is a useful tool for the design of polymer structures.

2.7.3 Star-like polymers and polymer brushes

In 2006, Kelly *et al.* used both CALB and *Rhizomucor miehei* lipase (RML) to produce ester-containing poly(ricinoleic acid) star polymers. There are two main approaches to synthesize such architectures: convergent ('arms-first') and divergent ('core-first'). In fact,

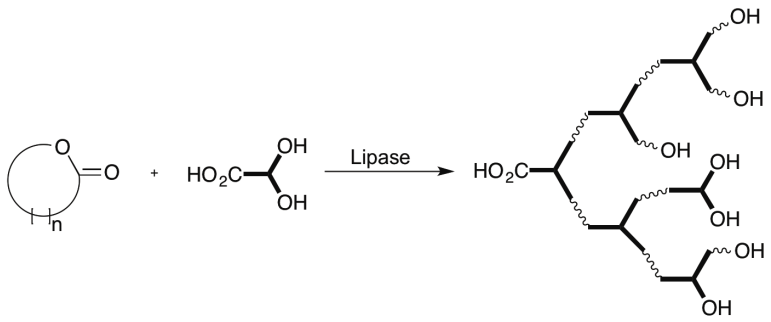
the group originally hypothesized that the divergent approach would be more successful, rationalizing that the architectures would grow faster and faster with the consumption of monomers. However, NMR analysis revealed that the convergent approach, which consists of attaching pre-synthesized polymers to a core, delivered the highest molecular weights. Both the polymerization and the attachment of arms to the core could be performed with CALB, while RML only catalyzed the second step of the convergent approach.[152] The resulting macromolecule showed high viscosity properties with low melting point temperatures, suggesting a potential use as biodegradable lubricants.

A Y-shaped miktoarm star polymer was recently synthesized using a combination of eROP and conventional atom transfer radical polymerization ATRP (Scheme 2.9).[153] The process involved modifying a CALB-catalyzed PCL into an ATRP macroinitiator in order to add two poly(glycidyl methacrylate) (PGMA) arms using a divergent approach. The resulting amphiphilic polymer self-assembled into different micellar morphologies with potential towards biomedical and industrial applications. Similar strategies have also been employed to produce H-shaped polymers.[154]



Scheme 2.9 Combination of eROP and ATRP for the synthesis of Y-shaped polymers.

Hyperbranched polymers were obtained for the first time by Skaria et al in 2002. They used a combination of eROP and AB_2 polycondensation (Scheme 2.10),[155] which were both catalyzed by CALB in one synthetic step. It was concluded that it is challenging to polymerize compounds that are only soluble in polar solvents where enzymes possess low catalytic activity. Perhaps using ionic liquids as a reaction media could solve this limitation. More recently, a cyclotetrasiloxane framework was used to synthesize oligoesters chemoenzymatically.[156] This synthesis was characterized by an impressive consumption of monomers (65-80 %) in a relatively short time period (24-48 h). In another example, PF lipase was employed to produce graft polymers of poly(lactide) via a divergent approach. Different initiators were used to obtain different types of branched and linear architectures. Exploring the enzymatic degradation of the structures with comparable molecular weights by hydrolysis revealed that higher number of branches enhances the degradability.[157]



Scheme 2.10 Synthesis of a hyperbranched polymer.[155]

Also another type of branched architecture, molecular brush, can be synthesized using convergent and divergent synthetic pathways. As mentioned above, a combination of both eROP and conventional ATRP can be used to produce amphiphilic products with different

degradation rates. Hans *et al.* demonstrated in 2007 that it is possible to produce heterografted molecular bottle brushes using a divergent approach. They took advantage of the partial substitution of hydroxyl groups by divergent eROP using CALB, after which the more efficient ATRP polymerization of methyl methacrylate (MMA) or n-butyl methacrylate (BMA) was then used to fill the initiator-functionalized sites in the main chain, as illustrated in Figure 2.6. This yielded structures with dispersities lower than 1.4.[158] To produce grafted surfaces, Tsuji *et al.* treated a prepolymerized poly(L-lactide) PLLA surface with an alkaline solution to reveal active sites, which are able to initiate the lipase-catalyzed polymerization of cyclic monomers. The polymerization of caprolactone monomers on PLLA yielded polymers with tunable degradation rates.[159]

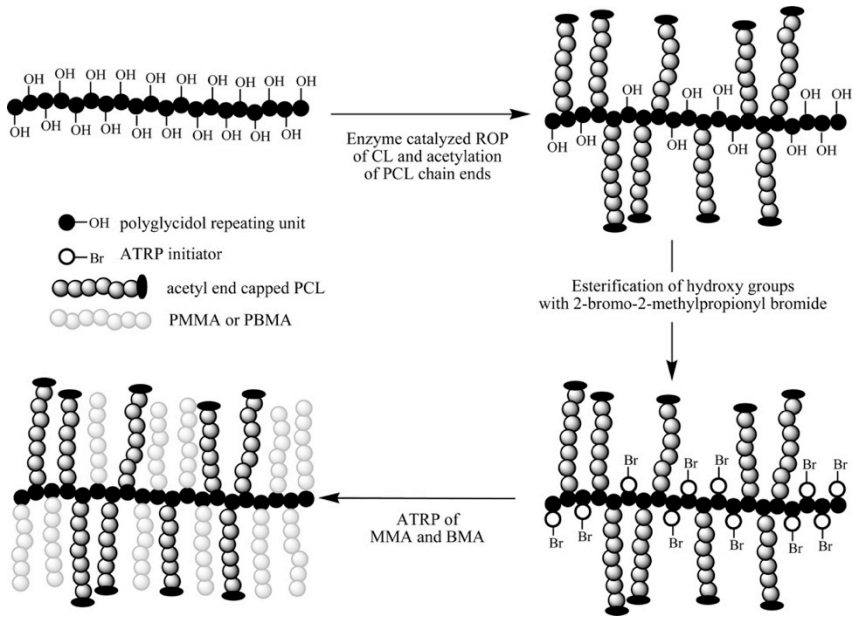


Figure 2.6 Preparation of heterografted brush copolymers by a combination of eROP of ε-CL and ATRP of MMA and BMA. Reproduced with permission.[158] Copyright © 2007, American Chemical Society.

Park *et al.* combined eROP grafting with organometallic catalysis.[160] After the organic synthesis of polyhydroxyaspartamide (PHEA), both CALB and tin octoate ($Sn(Oct)_2$) were employed for the ROP of *p*-dioxanone where the hydroxy moiety of PHEA served as macroinitiator. Although organometallic catalysis has some drawbacks, it is known to be very versatile and efficient. Nevertheless, CALB-catalyzed synthesis produced materials with higher crystalline order, better spherulitic morphology, and solubility characteristics $Sn(Oct)_2$. Keul and Möller likewise used a combination of organometallic and enzymatic catalysis (CALB) to obtain heterografted polymer brushes based on polyglycidols and PCL, and tested their degradation rates in order to tailor them to biomedical applications such as surgical sutures and drug delivery.[161] Barely no degradation was observed during three months and longer experiments would be needed.

2.7.4 Random and block copolymers

2.7.4.1 Copolymerization of cyclic species

Sobczak used three different lipases for the homo- and copolymerization of lactones with the goal of obtaining biodegradable polyesters that possess tunable degradation kinetics. In fact, glycolide (GL) was copolymerized with ε-CL and with racemic lactide (*rac*-LA), using PEG as a macroinitiator.[123] As expected, as the concentration of initiator decreased, the resulting molecular weight increased and the conversion showed a decreasing tendency. Increasing reaction temperature from 60 to 100 °C also increased the molecular weight and yield. Since the highest yields were observed on CALB, they further studied the copolymerization of cyclic species using this enzyme.[162] They reported once more that the eROP of more than one cyclic species at a time is possible using lipases. The study was

extended to combine the copolymers with 4,4'-methylenebis(phenyl isocyanate) (MDI) and successfully produced polyurethanes. In addition, Jiang *et al.* copolymerized two cyclic species to assess their thermal and crystalline properties in 2007.[163] After randomly copolymerizing ω-pentadecanolide (PDL) with *p*-dioxanone (DO), they observed that the larger PDL monomer is more reactive towards CALB. A higher fraction of PDL was therefore found in the final product (68 mol %), when equimolar monomer ratios were used. The resulting polymers obtained with different monomer ratios all showed high crystallinity and the crystal lattices possessed tunable degradation because of the large difference between the hydrophilicity of the monomers. Another pair of recently copolymerized lactones is 2-oxo-12-crown-4-ether (OC) with PDL.[164] Although both monomers possessed different rates of polymerization, random copolymers were obtained. Interestingly, the PDL segments in the polymer were able to crystallize, while the OC segments remained amorphous. The same research group further studied random copolymerization, but using CL instead of PDL. They reported easily tunable polyurethanes, possibly useful for soft tissue engineering applications because of their low crystallinity and increased hydrophilicity.[165]

2.7.4.2 Combination of eROP with eATRP

Atom transfer radical polymerization (ATRP) is a versatile technique that allows the polymerization of a wide range of monomers and some examples above already showed its potential in the synthesis of branched and brush-like polymer architectures. In a process resembling ATRP, vinyl monomers containing functional groups, such as amines, alcohol and epoxy groups, have been polymerized with metalloenzymes instead of transition metal catalysts.[166] Enzymatic ATRP, eATRP, combined with eROP was recently demonstrated for

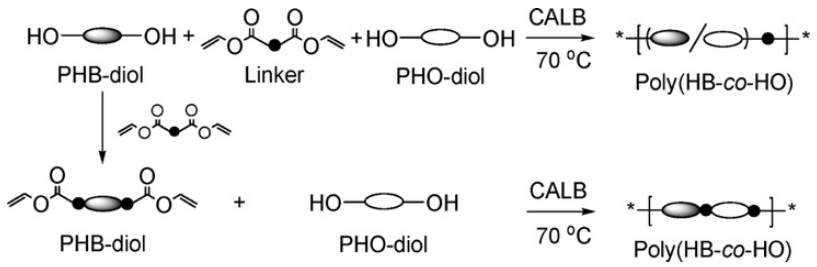
the first time by Zhou *et al.* using a synthetic peroxidase mimicking polypeptide bearing an iron porphyrin group. They showed that using renewable catalysts is a versatile approach for the production of biocompatible block copolymers.[167] After performing the eROP of ε-CL using an initiator containing a bromine end-group, this block then served as a macroinitiator to successfully polymerize glycidyl methacrylate by eATRP.

2.7.4.3 Block copolymers

Enzymatic block copolymerization is of interest in tuning material properties, particularly for biomedical applications. In an effort to develop block copolymers using eROP, de Geus *et al.* and Peeters *et al.* combined in 2005 sequential polymerizations in a one-pot cascade approach, yielding block copolymers.[113, 168] The bifunctional initiators, possessing a hydroxy and a bromine end-group, were reacted with ε-CL and MMA in consecutive and then simultaneous polymerization reactions, using CALB for eROP, and conventional ATRP. de Geus *et al.* found that the main factors influencing the copolymerization are the structure of the initiator and the amount of water present in the reaction medium, which pushed Peeters *et al.* to focus on only one initiator structure, 2-hydroxyethyl α-bromoisobutyrate. Recently, eROP was used to synthesize block copolymers for drug delivery applications from a renewable monomer, ω-pentadecalactone, again using CALB and mild organic chemistry.[169] Amphiphilic block copolymers and terpolymers were produced and they showed micellar self-assembly behavior, succesfully encapsulating Nile Red. In vitro degradation studies demonstrated a slow release of up to 8 hours. In another example of CALB-catalyzed block copolymerization, PCL-PEG-PCL triblock copolymers were synthesized in an environmentally friendly, one-pot biocatalytic route using PEG as a macroinitiator.[170] In this

study, the authors observed that varying the chain length impacts the crystallization behaviour, which is mainly determined by the PCL blocks, suggesting it inhibits the crystallisation of PEG. They furthermore discovered that different self-assembled morphologies and critical micellar concentrations could be obtained by varying the initiator:ε-CL ratio.

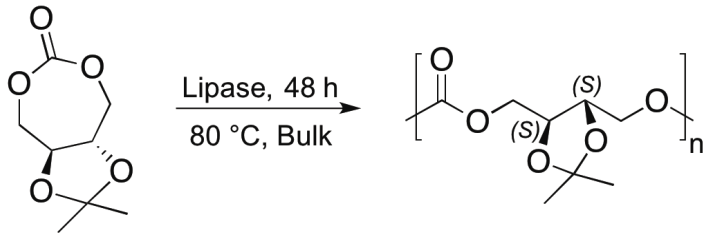
Block copolyesters have been used to produce thermoplastic materials via polycondensation of prepolymers with reactive divinyl adipate (Scheme 2.11).[171] The resulting materials posessed molecular weights of up to 25 100 g/mol and exhibited high melting point (136 to 142 °C) and low glass transition temperature T_g (-37 to -39 °C), which suggest potential as biocompatible and biodegradable materials.



Scheme 2.11 Synthesis of block copolymers by lipase-catalyzed polycondensation. Reproduced with permission.[171] Copyright © 2009, American Chemical Society.

CALB was successfully used to polymerize L-tartaric acid-derived monomer, (5S,6S)-dimethyl 5,6-O-isopropylidene-1,3-dioxepin-2-one (ITC, Scheme 2.12), designed to allow the functionalization of biodegradable thermoplastics after ketal group deprotection. Bisht *et al.* first polymerized this monomer using both lipases and chemical catalysts[172] and relatively high molecular weights (15 500 g/mol) but high dispersities (1.7) were obtained. Other lipases, such as PF, PS and PPL, did not produce polymers as efficiently. To tune the

biodegradability of polymers, ester functions were combined with carbonate functions by copolymerizing ITC with ε-CL. A tin catalyst was used for this 'one shot' block copolymerization step, where the monomers were fed simultaneously instead of sequentially.



Scheme 2.12 Lipase-catalyzed ring-opening polymerization of ITC.

2.8 Conclusions

Lipases are a benign alternative to organometallic catalysts in many syntheses as they are safe to use and can improve the biocompatibility of products by reducing toxic metal residues. In addition, the use of lipases follows several green chemistry principles. They are renewable, reusable and can function under mild conditions while also catalyzing stereo- and regiospecific reactions. Although enzymatic ring-opening polymerization (eROP) reactions have been extensively studied, the enzymatic lactonization of cyclic precursors and macrocyclic structures in particular is still a relatively new area. In this review, we have highlighted the important synthetic guidelines of enzymatic lactonization and eROP. Solvent polarity, concentration, type of lipase and water content are critical parameters to consider for enzymatic lactonization reactions. For eROP, the amount of enzyme used and reaction time are essential parameters to consider to control the molecular weight, conversion and dispersity, $Đ_M$, of polymers. As separation of the catalyst from highly viscous reaction media in

polymerizations is required and recyclability is desirable, one of the continuing challenges, particularly to reduce costs in large-scale production, is improving the immobilization and catalyst removal methods and mechanical stability of immobilization matrices.

The enzymatic synthesis of complex polymer architectures, such as stars, brushes, graft and block copolymers, is becoming increasingly popular as enzymatic techniques can combined with other types of polymerizations, such as atom transfer radical polymerization, ATRP. New synthetic engineered enzymes are emerging in this field. The combination of different methods allows producing polymers with several different functional groups, which further enables tuning the thermomechanical properties, self-assembling characteristics and degradation rate. As lipases are versatile catalysts, they have recently been explored in the synthesis of polyamides that would be difficult to produce by other methods or require harsh conditions.[133, 173-174] From environmental point of view, it is of interest to find an efficient way to degrade and recycle the produced materials. It has been shown that enzymatic degradation followed by repolymerization can generate polymers with molecular weights sometimes even higher than those of the original material. These methods are expected to gain more attention in future.

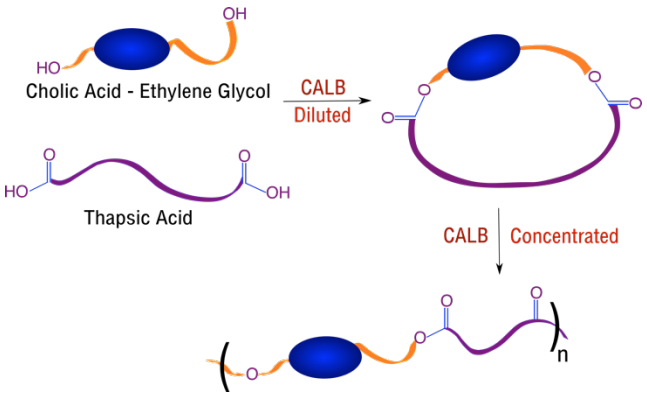
Chapter 3 - A Two-Step Enzymatic Synthesis of Biocompatible Polymers made from Cholic Acid[†]

Article

[†] Included with permission. (19) Champagne, E.; Lévaray, N.; Zhu, X. X., *A two-step enzymatic synthesis of biocompatible polymers made from cholic acid*. ACS Sust. Chem.& Eng. **2016**.

3.1 Abstract

Polyesters are known biodegradable materials that are frequently used for biomedical applications that require biocompatibility. Their synthesis usually requires transition metal catalysts, which may become a source of contamination. In addition, using such compounds translates to extensive purification procedures, which do not agree with green chemistry principles. In addition to being renewable, enzymes such as lipases are milder for biological systems, and were studied for both ring closure and ring opening reactions. Here, *Candida antarctica* lipase B was used in ring closure, reducing a 2-step synthesis to a single step with 58 % yield. The bile acid-containing macrocycles were subsequently polymerized with the same enzyme; relatively high molar masses (40 000 g/mol) were obtained. The conditions for the enzymatic ring closure and ring-opening reactions were established through the reaction of thapsic acid with 1,10-decanediol. The di- and tetralactones afforded semi-crystalline polymers with relatively high molar masses. Therefore, lipases were successfully used for both ring-closing reactions and ring-opening polymerizations of large rigid moieties as well as more flexible structures. The use of enzymes for the multistep syntheses shows their utility as a simple and green method for monomer and polymer synthesis with better biocompatibility and tunable properties.



Keywords: *Lipases, Bile acids, Macrocycles, Lactones, Biodegradable, Green chemistry*

3.2 Introduction

Polyesters such as poly (lactic acid) are widely used in biomedicine for bone fixation, ligament attachment, and as surgical sutures.[175] However, small acidic molecules formed upon degradation cause local acidity and inflammation.[176] To circumvent such problems, larger molecules such as bile acids (BAs) offer variable degradation kinetics, toughness, and flexibility.

BAs are natural compounds present in the human body as salts, whose function is the emulsification of triglycerides during digestion. They are promising precursors for the synthesis of biomedically relevant materials. Polymers made from BAs have been shown to possess shape-memory properties and glass transition temperatures (T_g) close to body temperature.[9, 13, 38, 177] In addition, varying the nature and length of the alkyl chain linker allows tuning of the degradation and thermo-mechanical properties of these materials.[12]

Previously, BA-based macrocyclic monomers and their polymers were synthesized through the use of Grubbs' catalysts for ring-closing metathesis (RCM) and entropy-driven ring-opening metathesis polymerization (ED-ROMP).[11-12] Hodge and Chakiri also performed such reactions on deoxycholic acid to produce functionalized polymers that could support cell growth.[178] The use of transition metal catalysts resulted in relatively high weight-average molecular weight (M_w) and yield, where the obtained homo- and copolymers displayed interesting thermo-mechanical properties.[9, 12]

The main challenge in the use of such heavy-metal catalysts is the full removal of trace amounts (in ppm) of these metals, which may be a safety concern for biomedical applications.[179] The purification procedures are laborious and costly, requiring much time and

energy. Therefore, greener and more sustainable synthetic methods are needed and the use of environmentally benign catalysts may alleviate such problems.[23]

The use of lipases is an alternative for the synthesis of polyesters, and the activity of such enzymes as catalysts has been extensively investigated.[24, 29] Several isoforms are now commercially available in the pure and immobilized forms. They are known to hydrolyze triglycerides in the body, but the reaction is reversible and may be used to perform esterification and transesterification reactions under laboratory conditions.[17, 115, 180] In fact, they have been particularly useful for the ROP of various monomers.[151, 181-183]

Candida antarctica lipase B (CALB) (EC 3.1.1.3) possesses many desirable advantages in the field of enzymatic catalysis. These include substrate promiscuity, combined with a high regio- and enantioselectivity, which makes the enzyme compatible with a multitude of substrates, but also limits side reactions.[61] CALB functions in harsh non-polar organic solvents and at temperatures of up to 90 °C.[13] The immobilized form of CALB (iCALB) has shown high potential, since it allows catalyst reuse with enhanced thermal stability. Immobilizing the enzyme also exposes its active site, increasing its propensity towards bulky substrates.[16, 67-68, 84, 88, 105, 151, 184] iCALB was shown to be an efficient catalyst for the esterification, transesterification, and lactonization of large flexible moieties.[48, 105, 116, 120] However, such reactions involving rigid and bulky molecules remain largely unexplored.

Previously, iCALB was used successfully in the enzymatic ring-opening polymerization (eROP) of BA-containing macrocycles.[13, 150] Although the preceding ring-closure reaction still required the use of Grubbs' catalysts, the new synthetic pathway allowed reduction of the heavy transition metal catalyst from 310 to 12 ppm.[151] Similarly, Hodge and

Chakiri used this enzyme for the eROP of a variety of BA macrocycles, and were able to measure the differing degrees of reactivity for the cleavage of the ester groups.[151]

It is logical and even necessary to consider macrolactonization reactions in the absence of heavy metals catalysts in the synthetic route. However, it is challenging to obtain cyclic monomers with rigid structures such as BAs, since restraining the linear alkyl chain linking both ends of CAEG is entropically costly.[185] Standard organic reactions such as the Steglich or Yamaguchi esterifications are not sufficient.[186] Several approaches to pre-organize the structures are possible,[187] but increase the reaction complexity and the number of synthetic steps required.

As enzymes are known for their specificity,[188] iCALB-catalyzed ring closing reactions have been considered. Indeed, the combination of enzymatic ring closing (eRC) and eROP of rigid macrocyclic monomers would offer an ideal solution to obtain more biocompatible polyesters. To our knowledge, only flexible aliphatic chains have been reported to macrocyclize this way,[49, 74, 105] but iCALB was found to be particularly suitable, as Zigolo et al. theoretically concluded with docking studies.[68] They found that the enzyme's active site can accommodates cholic acid (CA) moieties in such a way that it will selectively react with the extremities of the molecule, i.e., positions 3 and 24 (Scheme 3.2), demonstrating that iCALB is highly regio-selective.

Since the same enzyme can be used for both ring closing and ring-opening reactions, several parameters need to be considered in order to obtain the desired product. Key conditions include reaction time, enzyme and reactant concentrations, and solvent polarity, often denoted by the logarithm of the partition coefficient (log P), used as a quantitative measurement of the polarity of a solvent (its value increases with decreasing polarity).

In the case of esterification reactions, a large quantity of enzyme, or rather the high activity in propyl laurate units (PLU), leads to an increased reaction rate until a certain threshold, where the rate remains stable.[125-126] Therefore, when varying the amount of catalyst used while keeping a fixed reaction time, a linear increase in conversion can be observed. The concentration of enzyme needed is much lower in the case of eROP reactions, since using large amounts of catalyst results in lower M_w.[13] This was observed in the case of ε-CL by Deng et al. when they observed a decrease in molar mass with an increased amount of enzyme used. [66] Indeed, lipases target ester functions once the concentration of monomers is substantially decreased, which could be the cause for increased dispersity after long reaction times. As for the concentration of reactants, it should be kept low for cyclization reactions, while polymerization reactions typically need to be performed in concentrated conditions. Furthermore, a parameter that should be kept constant for both types of reactions is solvent polarity. In fact, low polarity is preferable when common organic solvents are used for lipase-catalyzed reactions where ester bonds are created. The use of such solvents tends to disrupt the nature of the active site by displacing the essential water layer and by interacting with its polar amino acid sequence.[18, 52, 127]

In this study, we used iCALB in both eRC and eROP steps involving large CA units, and optimized the reaction conditions to obtain polymers with relatively high M_w and good thermo-mechanical properties.

3.3 Experimental section

3.3.1 Chemicals and materials

Often, the amount of catalyst needed is expressed in relation to the weight of the reactants.[105] For macrocyclization reactions, we prefer to determine the amount of PLU used per mole of reactants. Hence, for the synthesis of monomers **3**, **4** and **7**, the amount of catalyst used was derived from a previous study.[115] The amount of PLU used per mole of substrate (multiplied by two, for two esterification reactions per macrocycle) was calculated and resulted in 517 g of iCALB per mole of diacid (286.41 g/mol). As for the eROP, they are expressed percentages, since they are used in catalytic amounts. Here, the amount of enzyme used for polymerization (in wt%) denotes a percentage of weight in relation to the weight of macrocyclic monomers used. As others found, the immobilized enzyme can be reused for many cycles.[88-89]

3.3.2 Methods

3.3.2.1 Catalyst concentration

Often, the amount of catalyst needed is expressed in relation to the weight of the reactants.[105] For macrocyclization reactions, we prefer to determine the amount of PLU used per mole of reactants. Hence, for the synthesis of monomers **3**, **4** and **7**, the amount of catalyst used was derived from a previous study.[115] The amount of PLU used per mole of substrate (multiplied by two, for two esterification reactions per macrocycle) was calculated and resulted in 517 g of iCALB per mole of diacid (286.41 g/mol). As for the eROP, they are expressed percentages, since they are used in catalytic amounts. Here, the amount of enzyme

used for polymerization (in wt%) denotes a percentage of weight in relation to the weight of macrocyclic monomers used. As others found, the immobilized enzyme can be reused for many cycles.[88-89]

3.3.2.2 Characterization

A Bruker AV-400 spectrometer possessing a 400 MHz field was used to obtain the Nuclear Magnetic Resonance (NMR) spectra. The elemental analysis was performed using a EAS-1108 Fisons dynamic flash combustion apparatus. Electrospray ionization (ESI) was used in order to analyze the samples by mass spectrometry. The gel permeation chromatography (SEC) was performed on a Breeze system from Waters, equipped with a 717 plus autosampler, a 1525 Binary HPLC pump and a 2410 refractive index detector using three consecutive columns. A TGA 2950 and a DSC Q1000 from TA instruments were used to determine the degradation, melting, crystallization and glass transition temperatures. TGA experiments were performed with a heating rate of 5 °C / min until 600 °C was reached. The DSC runs were performed with a temperature modulation (amplitude of 0.95 °C) in order to differentiate reversible from non-reversible transitions. A temperature range from -60 °C to 140 °C was covered.

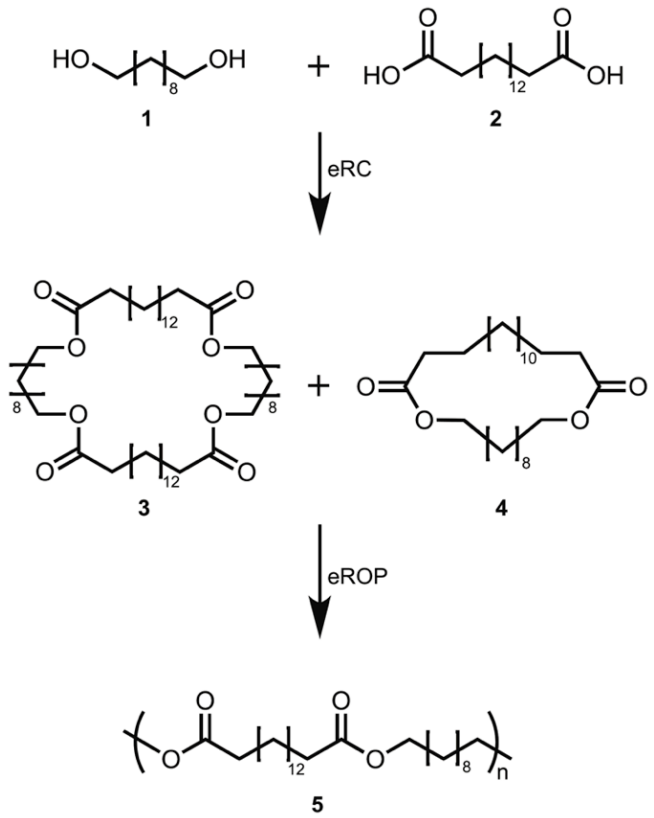
3.3.2.3 Synthesis of monomers 3 and 4 (Scheme 3.1)

To a solution of TA (500 mg, 1.7 mmol) in 170 ml cyclohexane (0.01 M) was added iCALB (900 mg) and 1,10-decanediol (300 mg, 1.7 mmol). The solution was then heated to 70 °C and mechanically stirred for 48 h before filtration and evaporation under reduced pressure. The resulting dilactones 1,14-dioxycyclooctacosane-2,15-dione, and tetralactones 1,28-

dioxycyclohexapentacontane-2,29-dione (monomers **3** and **4**), were quantitatively obtained as a white powder after drying under vacuum.^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 4.15-4.05 (4 H, m, CH_2), 2.33 (4 H, m, CH_2), 1.42-1.23 (8 H, m, CH_2), 1.69-1.60 (32 H, m, CH_2). ESI-MS: *m/z* 425.361 ($M + H^+$), 447.343 ($M + Na^+$), 871.700 ($M + Na^+$).

3.3.2.4 Synthesis of polymer 5

iCALB and the obtained mixture of monomers **3** and **4** were dried over P_2O_5 in a desiccator under vacuum for 24 h before use. A solution of monomers **3** and **4** (200 mg, 0.47 mmol, calculated from the molar mass of monomer **4**) in toluene (1.2 ml, 0.4 M) was added to a vial containing iCALB (1.0, 5.0 or 10.0 mg) with a syringe. The mixture was allowed to stir magnetically for 24 or 48 h at 70 °C in a vial holder on a hot plate. Small amounts of toluene (0.10 ml) were added to the reaction mixture as the viscosity increased with time. 5 ml of $CHCl_3$ and 5 g of Na_2SO_4 were added to end the reaction before filtering out the enzyme. After evaporating the solvent under reduced pressure, the polymer mixture was re-precipitated by dissolving it in 5 ml THF and adding it drop-wise to 80 ml of hot ethyl acetate. The mixture was left to cool down at room temperature for 4 hours before collecting the solids by filtration.



Scheme 3.1 eRC of 1,10-decanediol with **2**, and eROP of monomers **3** and **4**.

3.3.2.5 Synthesis of cholic acid-ethylene glycol ester (CAEG) 6

A previously established protocol was followed and slightly modified.[189] To a solution of CA (20 g, 49.0 mmol) in 90 g ethylene glycol was added 7-8 drops concentrated H_2SO_4 as catalyst. The mixture was stirred for 5 h at 60 °C and poured into 0.4 L cool brine after cooling to room temperature. The mixture was left to precipitate overnight and further cooled in an ice bath before vacuum filtration. The solid was washed with 1 M solution of $Na_2CO_{3(aq)}$ and H_2O and then dried under vacuum. The white powder was obtained by recrystallization in a mixture of EtOH and H_2O (18.0 g, 81 %). ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 4.80-4.76 (1 H, m, 3-OH), 4.33-4.30 (1 H, d, J = 4.31 Hz, 7-OH), 4.13-4.10 (1 H,

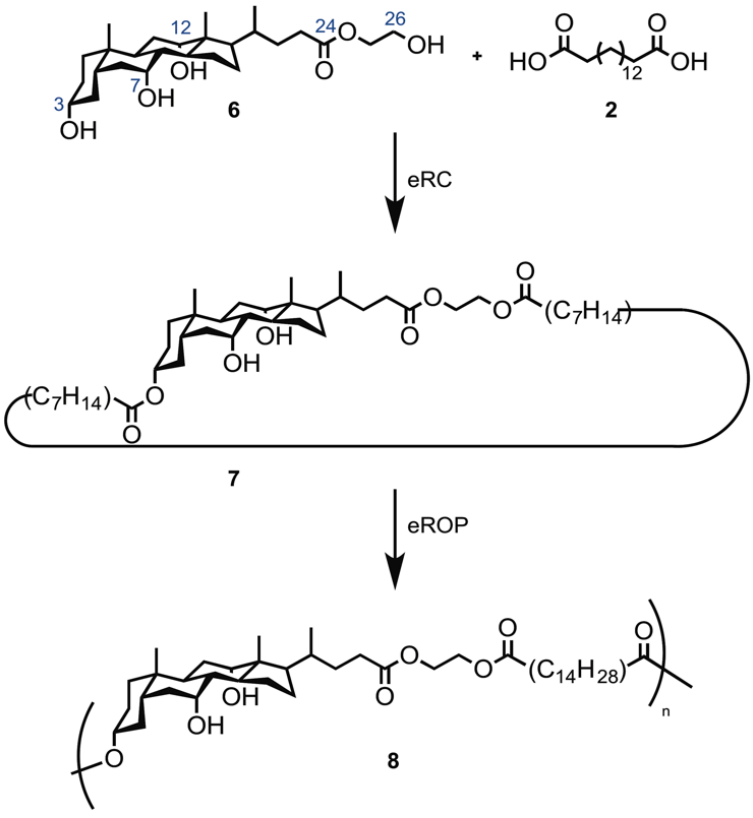
d, J = 3.48 Hz, 12-OH), 4.03-3.99 (2 H, m, 25-CH_2), 3.81-3.76 (1 H, m, 12-CH), 3.64-3.60 (1 H, m, 7-CH), 3.55 (2 H, m, 26-CH_2), 3.22-3.13 (1 H, m, 3-CH), 0.93 (3 H, d, J = 6.14 Hz, 21-Me), 0.81 (3 H, s, 10-Me), 0.59 (3H, s, 13-Me).

3.3.2.6 Synthesis of monomer 7

As shown in Scheme 3.2, to a solution of TA (320 mg, 1.12 mmol) in 220 ml toluene was added iCALB (670 mg). The solution was then stirred and heated to 70 °C before adding CAEG (500 mg, 1.1 mmol). The mixture was left to react for 10 to 48 h, as indicated in Table 2.2, before being diluted with $CHCl_3$ (100 ml) and filtered. The solids were washed with $CHCl_3$ and the filtrate was dried under reduced pressure. The pale yellow gum was dissolved in a small amount of DCM and passed through a patch of basic alumina, and then isolated by silica column chromatography with DCM as the mobile phase, leaving the partially reacted molecules behind to yield a clear gum, macrocycle **7** (57.6 % by LC-MS; 270 mg, 35.7 % isolated). Elemental analysis (Found: C, 71.3; H, 10.5. Calc. for $C_{42}H_{70}O_8$: C, 71.8; H, 10.0 %), ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 4.48-4.39 (1 H, m, 3-CH), 4.21 (4 H, m, 25-26-CH_2), 4.11 (1 H, d, J = 3.59 Hz, 12-OH), 4.01-3.98 (1H, d, J = 3.30 Hz, 7-OH), 3.79-3.73 (1 H, m, 12-CH), 3.64-3.60 (1 H, m, 7-CH), 1.26 (24 H, m, J = 7.33 Hz), 0.94 (3 H, d, J = 6.24 Hz, 21-Me), 0.83 (3 H, s, 10-Me), 0.60 (3 H, s, 13-Me). ESI-MS: *m/z* 720.542 (M + NH_4^+), 725.499 (M + Na^+).

3.3.2.7 Synthesis of polymer 8

A solution of monomer **7** (150 mg, 0.180 mmol) in dry toluene (0.50 ml, 0.40 M) was added to a vial containing iCALB (7.80 mg). The mixture was stirred at 70 °C for 10, 15, 24 or 48 hours, while adding small amounts (0.10 ml) of toluene to reduce the viscosity, every 8 hours. 10 ml of $CHCl_3$ were then added before filtering out the enzyme. The solvent was completely evaporated under reduced pressure, and the pale yellow solid was dissolved in 5 ml DCM. This solution was added drop-wise to 60 ml EtOH at 0 °C and left to precipitate with agitation for 4 hours before filtering and drying the white solid under vacuum.



Scheme 3.2 eRC of CAEG **6** with TA **2** and eROP of macrocycle **7** to obtain polymer **8**.

3.4 Results and discussion

Although the eROP reactions have been tested and optimized,[4,19] the conditions for eRC reactions are not well examined. To study the feasibility of performing a double esterification for eRC in one synthetic step using iCALB, the eRC reactions of simpler macrocyclic structures (28 and 56 atoms, monomers **3** and **4,** Scheme 3.1) without a bulky moiety were tested. Afterwards, the eROP of these monomers with different reaction times and catalyst amounts were examined in order to assess the impact of these parameters on the M_w (Table 2.1).

The optimized conditions were used for the eRC of bulky, macrocyclic molecules containing CA, yielding 34 atoms-long CA-bearing macrocycle **7**. The eROP of the newly obtained macrocycles was then performed using a similar procedure as in a previous report,[13] while optimizing the reaction time.

3.4.1 Synthesis of monomers 3 and 4

A one-step procedure was elaborated for the eRC of TA with 1,10-decanediol (Scheme 3.1, based on previous work.[49, 74, 105, 190] The lactones were formed in high dilution conditions (0.01 M) and in dry solvents of low polarity such as cyclohexane and toluene.

Mass spectrometry (MS) revealed a mixture of dilactones **4** (28 atoms-long) and tetralactones **3** (56 atoms-long). The results agree well with previous work, in relation to the variety of species obtained when using chemoenzymatic routes in the context of ring-chain equilibrium.[98-99] Very high yields (>95%) were obtained in both solvents. The product obtained in toluene required a purification step, while quantitative yields were obtained in

cyclohexane. This was not surprising; given the low solubility of the reactants in toluene, and the lower log P of cyclohexane.[127]

3.4.2 Synthesis of polymer 5

Unsubstituted macrocyclic esters possessing small ring strain are said to be the worst candidates for conventional anionic ROP.[191] However, their behavior is quite different for eROP. Indeed, for the eROP of the mixture of monomers **3** and **4** (Scheme 3.1), size exclusion chromatography (SEC) revealed Polymer **5** with satisfactory dispersities ($Đ_M$) of about 1.4 and relatively high degrees of polymerization (DP_n) of up to 94 units (Table 3.1). As can be observed from the same table, using 5.0 wt% catalyst for 24 h allowed the highest M_w (40 000 g/mol).

As expected from its linear stackable molecular structure, Polymer **5** is semi-crystalline with a melting temperature (T_m) and a crystallization temperature (T_c) of about 84 and 72 °C, respectively (Figure 3.1). These properties were found to be constant when changing reaction times (RTs) and catalyst concentration (Table 3.1). The T_g could not be observed on the DSC thermogram, the transition probably being too small to detect.

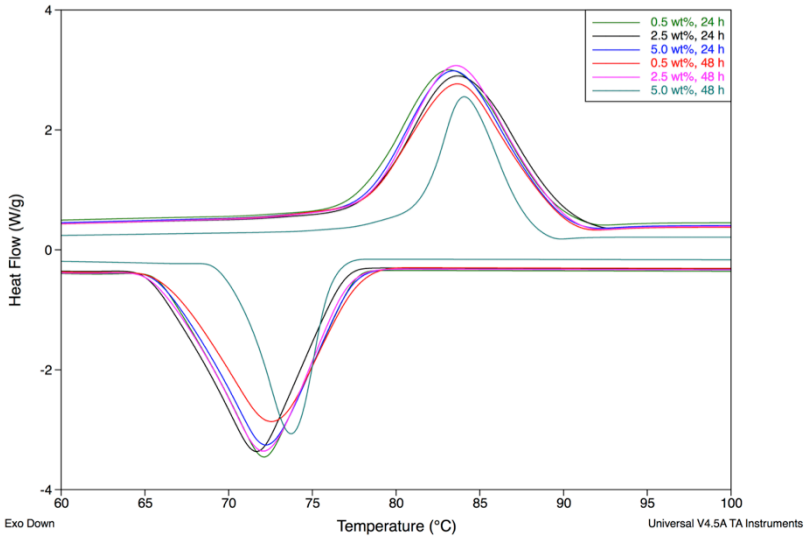


Figure 3.1 DSC curves showing the melting and crystallization temperatures of Polymer 5, with various reaction times and amounts of CALB used.

Satisfactory M_w, compared to the literature,[20] were measured when only 0.5 wt% iCALB was used (Table 3.1). However, the largest M_w values were obtained with 5.0 wt% enzyme and 24 h of reaction time. With fixed reaction time of 24 h, an increase in M_w was observed with increasing amounts of enzyme, with a high correlation coefficient (R^2) of 0.99 (Figure 3.2). Although eRC and other esterification reactions require the use of large amounts of catalyst, it is preferable to use much less in the case of eROP.[66, 123, 131, 161] Large amounts of catalyst favor maximum consumption of monomers, but results in many short polymer chains (high $Đ_M$) as was previously observed with 10 wt%.[13]

Table 3.1 Reaction time and enzyme amount for the synthesis of polymer 5

Time (h)	iCALB (wt %) [a]	M_w (g/mol) [b]	$Đ_M$ [b]	DP_n [b, c]	T_d (°C) [d]	T_c (°C) [e]	T_m (°C) [e]
24	0.5	30 200	1.42	71	362	72	84
	2.5	34 600	1.40	81	365	72	84
	5.0	40 000	1.48	94	367	72	83
48	0.5	32 200	1.47	76	365	74	84
	2.5	33 700	1.42	79	365	72	83
	5.0	37 800	1.46	89	367	73	84

[a] The amount of of iCALB used (wt % of the monomer used). [b] Weight-average molecular weight (M_w), dispersity ($Đ_M$), and degree of polymerization (DP_n) determined by SEC in $CHCl_3$ against polystyrene standards. [c] DP_n determined by using 424.7 g/mol as the monomer M_w (dilactone). [d] Degradation temperature (T_d) taken from the onset of the thermogravimetric analysis (TGA) thermogram. [e] Crystallization (T_c) and melting temperatures (T_m) taken from the differential scanning calorimetry (DSC) thermogram.

During the reaction, hydrolysis may occur, depending on the amount of water contained in the medium. Some groups simply observed a decrease in kinetics,[66, 118] while we observed a shift in equilibrium, towards oligomerization, as previously reported.[13, 24, 126] Here, shorter polymer chains formed when the reaction time was doubled from 24 to 48 h. As expected, this decrease in M_w was larger when more catalyst was used in the medium (Figure

3.2), since equilibrium could be reached faster. For the lowest concentration, 48 h produced a longer polymer, suggesting the reaction had not yet reached equilibrium at 24 h. Therefore, a reaction time longer than 24 h is needed for a substantial conversion of monomer, with such low iCALB: monomer ratio. For enzyme concentrations of 2.5 and 5.0 %, the trend is as expected, where the M_w decreases after prolonged reaction times.

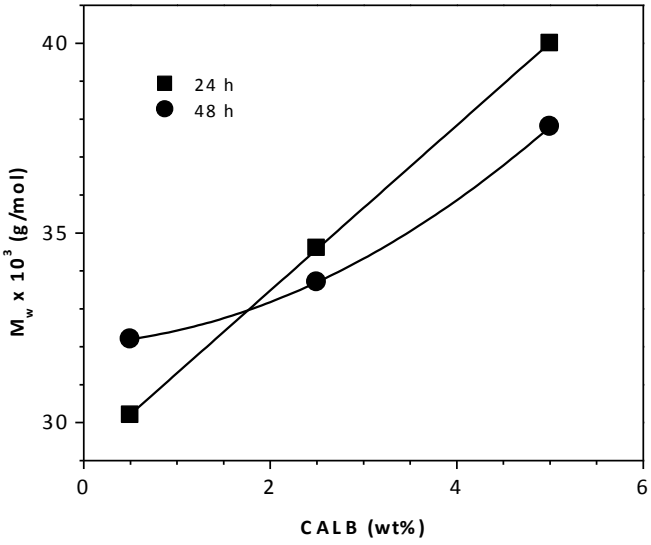


Figure 3.2 The influence of the amount of enzyme used (wt%) and of the polymerization time (squares, 24 h; circles, 48 h) on the M_w of polymer **5.**

3.4.3 Synthesis of monomer 7

iCALB catalyzed the combination of CAEG and TA in toluene at 0.005 M concentration, yielding macrocyclic structure **7** possessing three ester functional groups (Scheme 3.2). As in the case of monomers **3** and **4**, two ester bonds are formed in one synthetic step, with reusable enzymes as catalysts. To obtain the macrocycles in satisfactory

yields (58 %), the reaction conditions required high dilution (0.005 M) in low polarity solvents (high log P), relatively mild reaction temperatures (70 °C) and a longer reaction time (48 h). Side-product scavenging further allowed the equilibrium to shift towards the formation of ester bonds, rather than their hydrolysis.

The optimal concentration for eRC of the CAEG-containing monomer was found to be slightly lower than in the case of monomers **3** and **4**. Indeed, 0.01 M gave very low yields of cyclic species with a multitude of side products, while further dilution to 0.005 M gave the best yields. However, further lowering the concentration to 0.0025 M slowed the reaction kinetics significantly, since reactants were still observed after 48 h.

An excess of acyl donor for enzymatic esterification may prevent hydrolysis,[108-109] but no significant increase in yield was observed when using 5 equivalents of TA. In addition, an equimolar ratio of reagents is preferred in the case of lactonization to prevent the formation of linear oligomers. Hence, transesterification occurred between ethylene glycol and TA as evidenced by LC-MS analysis, resulting in ca. 11 wt% of linear ethylene glycol ester of TA.

The cyclic structure was confirmed by the use of three methods. ^{1}H NMR showed a shift of the proton at position 3 (Scheme 3.2) from 3.2 to 4.4 ppm, proving that an ester bond was formed. Furthermore, the large downfield peak representing carboxylic acids disappeared. See supplementary information for more details. Elemental analysis gave accurate results, with errors ≤ 0.5 %. Both liquid chromatography-mass spectrometry (LC-MS) and electrospray ionization-mass spectrometry (ESI-MS) gave an exact mass of 702.51 g/mol after subtracting the adduct (Na^{+}). These results demonstrated that unlike monomers **3** and **4**, only one cyclic species was formed when CAEG was used as the diol.

To our knowledge, it is the first time that a rigid molecule such as the cholic acid derivative CAEG is macrocyclized through the use of an enzyme in the absence of metal catalysts, linking both ends in a one-step procedure with TA. It is to be noted that the eRC of CA-containing molecules does not require the presence of C=C double bonds as required in metathesis reactions. This reduces the amount of synthetic steps required, enlarges the repertoire of the compounds that may be used in this synthesis, and may enhance both the stability of the reactants and of the final product.

3.4.4 Scavenging of side-products

Since CALB's natural action on an ester bond is hydrolysis in aqueous media, dry conditions should be maintained to allow a maximal amount of esterification reactions. Previous work showed that adding molecular sieves to the reaction medium during the reaction increased the yield of esterification reactions significantly.[115] We added a small amount of molecular sieves in the reaction mixture (5-6 beads for 1.1 mmol CAEG) and the yield increase from 44 to 58 %, as determined by LC-MS analysis. But larger amounts (100 g/L) of molecular sieves used did not improve the situation, probably due to the release of fine particles from the beads. This seemed to favor oligomerization rather than cyclization, as the viscosity increased with time.

A yield of 58 % was obtained by LC-MS when analyzing the crude product. The purification protocol may be further improved, since 36 % isolated yield was obtained.

The difference between the yields obtained for monomers **3**, **4,** and **7** may be due to the bulkiness and rigidity of the steroidal moiety in CA, compared to flexible chains in monomers **3** and **4**, which may significantly slow down the kinetics of cyclization. In fact, the

CA molecules possess different affinities towards iCALB, due to their different polarity and size and the active site of CALB is buried at the bottom of a mostly non-polar, narrow funnel.[15, 69] Furthermore, the precursors of monomers **3** and **4** retain most of their high flexibility upon cyclization, but TA is less flexible when restrained on both ends of CAEG. Thus, the formation of monomer **7** suffers from a higher entropic cost of cyclization.

3.4.5 Solvent choice

The choice of solvent for enzymatic catalysis is crucial. Larger log P values lead to easier esterification, and several hypotheses have been proposed for why this is. Indeed, when hydrophilic solvents are used, deactivation of the enzyme may be caused by the displacement of the water layer, vital to enzyme activity.[18, 127] It was also proven that strong interactions form between hydrophilic solvents and the enzyme's polar amino acid groups located at the active site, which could in turn change its catalytic activity.[52]

For the synthesis of monomer **7,** several runs were first performed in 1,4-dioxane, since the reactants were soluble in it. We were unable to isolate a satisfactory amount of the cyclic product, as others found previously.[18] Both toluene and cyclohexane as solvents afforded monomers **3** and **4**, and toluene was found to be optimal in the case of monomer **7**. Indeed, CAEG and TA showed very low solubility in cyclohexane, while hot toluene allowed the starting reactants to dissolve. Thus, a balance must be reached between the polarity of the solvent and the solubility of the reactants to obtain a higher esterification yield.

3.4.6 Synthesis of polymer 8

The eROP of monomer **7** was performed following our previous procedure for a macrocycle of similar structure, with a larger ring-size (38 atoms-long). In the present case, colorless and amorphous polymers with large M_w of up to 40 100 g/mol were obtained. This is a net increase over to the ones we obtained with RCM-catalyzed monomers previously.[13] The SEC data also revealed $Đ_M$ values ranging from 1.27 to 1.44 (Table 3.2).

In a previous study by Hodge et al., reaction times of 10 and 20 hours were used for the eROP of similar macrocyclic monomers.[151] It was determined that long reaction times are often not necessary to obtain polymers with M_w > 10 000 g/mol. This was therefore investigated for the eROP of monomer **7** (Table 3.2). Reaction times of 10, 15, 24 and 48 h were tested, while all other parameters were kept constant. The highest M_w were obtained at 15 and 24 h, with DP_n of 56 and 55, respectively.

In the beginning, when the reaction time increased from 10 to 15 h, the DP_n of Polymer **8** increased from 46 to 56. As discussed in the case of Polymer **5**, after a certain reaction time, the polymer M_w decreases due to hydrolysis of the polymer chains, once the concentration of monomer becomes very low and the ring-chain equilibrium is reached. This trend was observed here after 24 h reaction time (Table 3.2).

Table 3.2 The effect of reaction time on the polymerization of monomer 7

Time (h)	Conv. (%) [a]	M_w (g/mol) [b]	DP_n	$Đ_M$ [b]	T_d (°C) [c]	T_g (°C) [d]
10	42	33 300	47	1.27	344	12
15	50	40 100	57	1.41	341	12
24	57	39 500	56	1.44	342	14
48	47	33 000	47	1.42	344	8

[a] Conversion to Polymer **8** determined after precipitation. [b] Measured by SEC in THF against polystyrene standards. [c] T_d obtained from the onset of the TGA thermogram. [d] T_g obtained from the onset of the DSC thermogram.

Compared to the polymers made previously,[13] the T_g values of the polymers here are lower (Table 3.2 and Figure 3.3), while degradation temperatures remain similar (Table 3.2). The small differences in chemical structures seem to have an effect on the thermo-mechanical properties of the polymers. In fact, the difference between these polymers and the previous is the presence of a double bond and a small variation in the length of the alkyl linker.

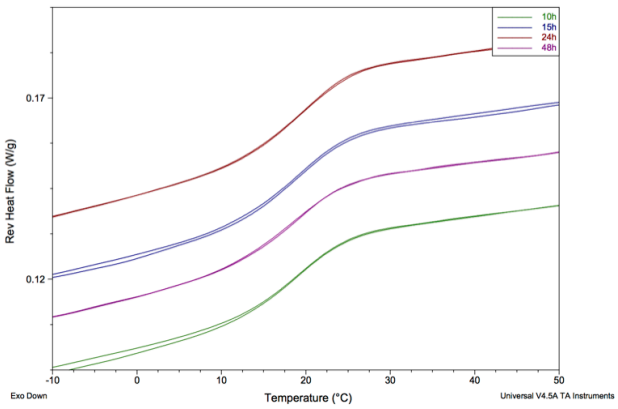


Figure 3.3 DSC curves showing the glass transitions for Polymer **8**, obtained with different reaction times.

3.5 Conclusion

Lipases are not only useful for eROP, but also for lactonization reactions, to obtain cyclic precursors. Indeed, eRC is possible, even for bulky substrates under high dilution, anhydrous, and low polarity conditions. It is the first time a synthetic procedure for the macrocyclization of bulky rigid molecules possessing both hydroxyl and carboxylic acid groups is established using an enzymatic catalyst. This work shows that the need for metal-based catalysts may be completely eliminated in the synthesis of BA-based biodegradable materials. The polymers obtained are free of metal contaminants, and the synthetic procedure is mild and easy to perform, providing a greener process. By exploiting the selectivity of enzymes towards BAs, the variety of monomers could be expanded to several other types of BAs and biological compounds to generate a large number of polymers possessing varying thermo-mechanical properties. Copolymerization could also offer a solution to tune such properties to suit various biomedical applications.

Supporting Figures and Information for Chapter 3

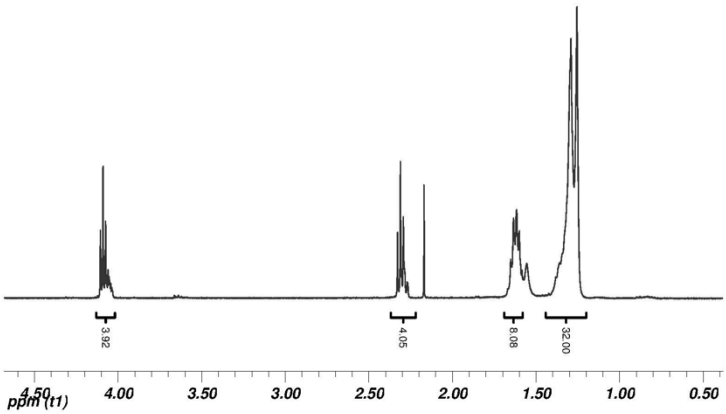
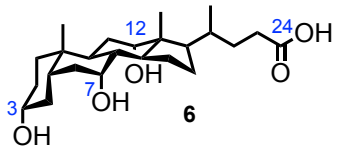


Figure 3.4 ^{1}H-NMR spectrum of monomers **3** & **4** in $CDCl_3$ at 400 MHz (compounds insoluble in DMSO).



Scheme 3.3 Numbering in the structure of cholic acid.

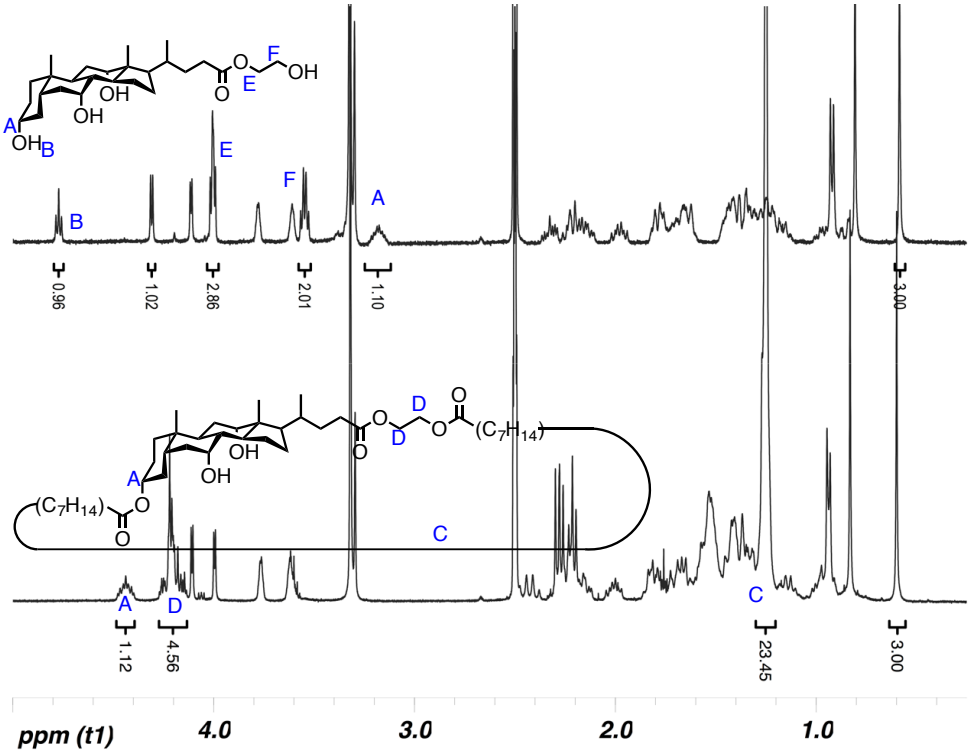


Figure 3.5 ^{1}H-NMR spectra of monomer **7** (bottom) and of its precursor CAEG **6** (top).

Figure 3.5 shows a clear shift of the signal of proton A located at position 3 on cholic acid (Scheme 3.3) from 3.2 ppm to 4.4 ppm, confirming the transformation of the esterification of the nearby hydroxyl group. The disappearance of signal of hydroxyl proton B at about 4.8 ppm also confirms the esterification. Furthermore, the two pairs of protons (E and F) at about 3.6 and 4.0 ppm in the precursor became equivalent with cyclization and are now located at 4.2 ppm (protons D) with an integration of 4.56 (with presence of overlap). Proton signals C at about 1.2 ppm with an integration of 23.45 are attributed to the 24 equivalent protons on 12 of the 14 CH_2 groups in thapsic acid.

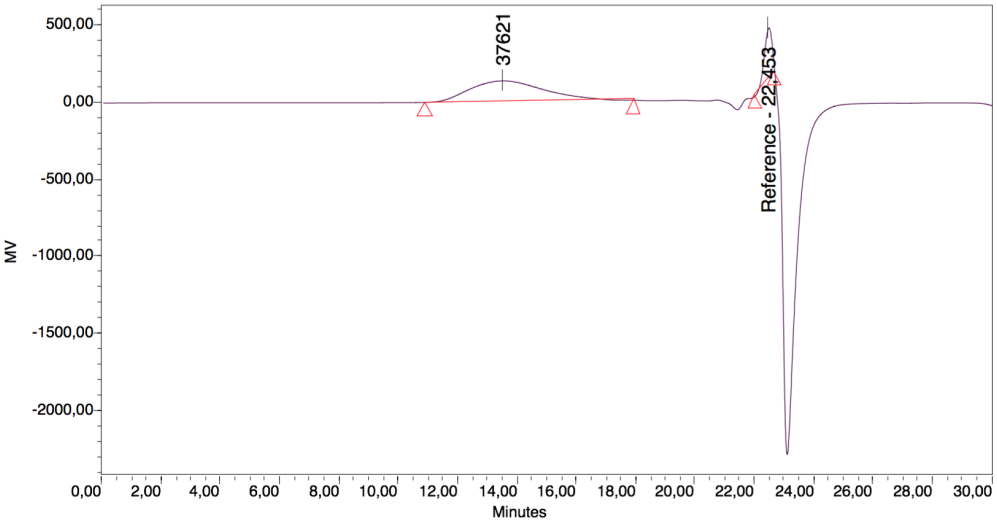


Figure 3.6 An example of a GPC curve obtained for polymer **5** (reaction time of 24 h and 5 wt% CALB as catalyst). The reference sample used in this curve is a polystyrene standard. The numbers shown are the peak values of the

Chapter 4 - Conclusions

An array of possibilities in the fields of polymer and bioorganic chemistry is enabled because of this work, since it is the first time such bulky and rigid molecules are macrocyclized using a lipase. Indeed, terminal double bonds are no longer required for cyclization of rigid moieties such as bile acids. This permits a larger freedom of choice in terms of substrates that can be polymerized. Since lipases create ester bonds, moieties, such as hydroxyl, ester, and carboxylic acid groups, are sufficient for cyclization, and hence for polymerization to occur.

Also, the fact these catalysts are natural, renewable, reusable, and non-toxic offers a clear advantage over organo-metallic catalysts. They are more sustainable, both monetarily and environmentally. Below is a more detailed outline of what was accomplished during this study.

4.1 eRC and eROP of flexible structures

A series of experiments was performed in order to confirm that iCALB is a good fit for the one-pot cyclization of large molecules. To simplify the initial experiment, 10 and 16 carbons-long mobile chains were used. Indeed, TA and 1,10-decanediol were chosen, and using different solvents and substrate concentrations, concepts were understood, and optimal conditions were determined. As expected, using cyclohexane as the solvent resulted in higher yields than when toluene was used, for solvent polarity reasons. The substrate concentration mostly affected kinetics at high dilutions. It was thus shown by ESI-MS that practically quantitative yields of macrocyclic structures were formed at a concentration of 0.01 M in

cyclohexane. Both 28 and 56 –long macrocycles were obtained, which confirmed that iCALB is capable of forming macrocyclic structures from two distinct molecules, in one pot. This also confirmed iCALB's tendency to catalyze the formation of various sizes of cyclic structures from the oligomers obtained during the reaction.

The di- and tetralactones were subsequently polymerized using different concentrations of iCALB, and reaction times of 24 or 48 hours. Results demonstrated that a reaction time of 24 hours was just enough to obtain a M_w of 40 000 g/mol when 5.0 wt% enzyme was used. Indeed, after 48 h of reaction time, the M_w decreased, which showed that hydrolysis might occur after the reaction reaches equilibrium. Furthermore, as the concentration of enzyme used increased, the M_w followed the same trend when using a fixed reaction time, which was as expected.

Based on DSC results, the polymers are semi-crystalline, with crystallization and melting temperatures around 72 °C and 83 °C, respectively. Relatively good control of the dispersity was also possible, obtaining $Đ_M$ of about 1.4. These results compare well to current literature results, where M_w are generally lower and $Đ_M$ values higher.

Synthesizing these polymers allowed a better understanding of CALB's mechanism, which proved useful for following similar synthesis, using bile acids.

4.2 eRC and eROP of bile acid-based structures

With the confirmation that iCALB accommodates large, mobile structures, we wanted to determine its efficiency for cyclization towards rigid moieties such as CA. After a

simple modification that consisted of adding ethylene glycol to the primary hydroxy site on CA, CAEG was successfully cyclized, using TA as a linker, in only one step. It was the first time that such a bulky, rigid structure was macrocyclized using lipase. Several characterization techniques were used to confirm the new macrocyclic structure: ^{1}H-NMR, elemental analysis, and MS. LC-MS was used to determine the exact yield.

Several parameters were important to consider towards obtaining the right product with an acceptable yield. For one, maintaining equilibrium between solubility of the monomers and the polarity of the solvent was preferable for lactonization to occur. Hence, toluene was the best fit as a solvent. We also found that the optimal reactant concentration and reaction time varied with monomer type, as 0.005 M and 48 h gave the best yields for this reaction. Maintaining an anhydrous environment also played a considerable role towards the amount of ester bonds formed.

As mentioned in Annex 1, other synthetic methods were proposed and tested, but cyclization of such rigid moieties presents a considerable challenge, entropically. As observed, iCALB is specific enough to catalyze such a reaction.

The bile acid-containing cyclic moieties were further polymerized (eROP) using iCALB, which also yielded high M_w (up to 40 100 g/mol) and low $Đ_M$ (around 1.4), compared to previous work. As in the case of polymer **5**, long reactions times of 48 h were detrimental for the polymer's M_w. Moreover, the colourless polymer is completely amorphous, as DSC measurements confirmed, with a T_g of about 12 °C. Given that functionalization and varied architectures allow to obtain polymers with a larger T_g, this polymeric material is a promising candidate for biomedically relevant compounds such as sutures, stents and tissue regeneration media.

4.3 Future work

By exploiting the selectivity of lipases towards functional groups, the scope of monomers may be expanded to other biological compounds to generate a variety of other new polyesters. This represents a general solution to biocompatibility issues related to the bioorganic synthesis of macrocycles and polymers and may have implications for the use of other enzymes in the making of polymers.

In the future, copolymerization of monomer **7** with other cyclic species could help tune thermo-mechanical properties for specific health-related applications.[163] In fact, varying the ratio of mobile vs. rigid moieties in the material normally causes a change in the material's flexibility and strength. As an example, the cyclization and polymerization of fatty acids is a field that still remains to be explored, and that has great potential given how accessible these molecules are.

Proteases may also be useful to create amide bonds. They have previously been used before as polymerization catalysts,[42] and exploiting their amidation capabilities could offer more possibilities in terms if small-molecule bioorganic reactions.

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Appendix 1 – First Attempts at Cyclization

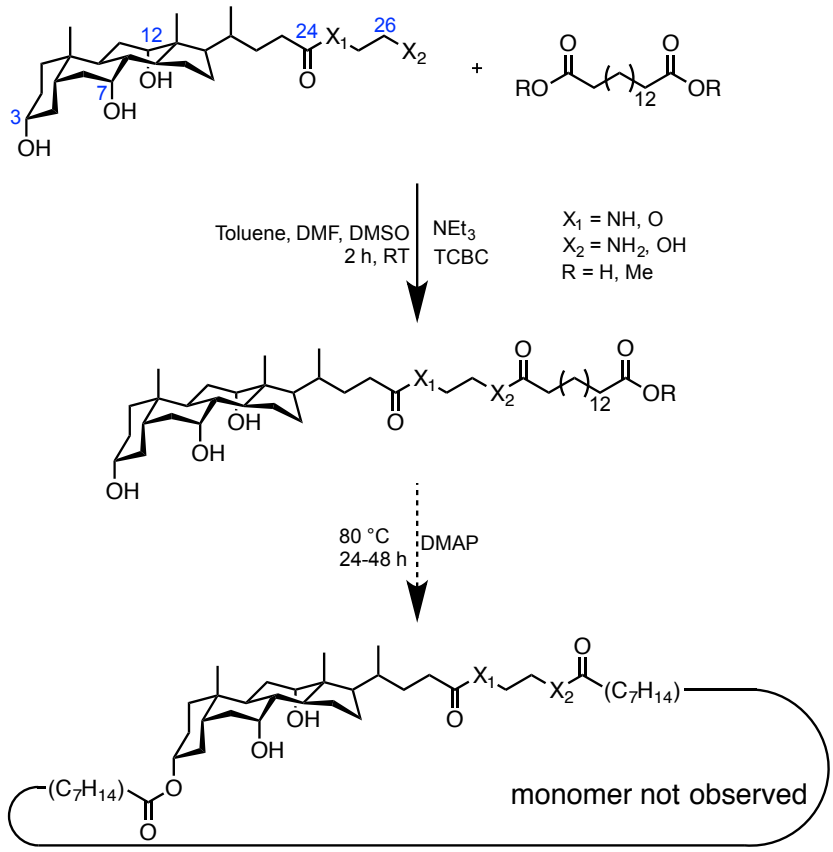
The following information was not disclosed in the results article (Chapter 3), but could be pertinent for researchers working on macrocyclization reactions.

Before exploring enzymatic catalysis, efforts were made to perform amidation and esterification reactions using more conventional organic methods. Here is an outline of what was done.

A1.1 Yamaguchi esterification

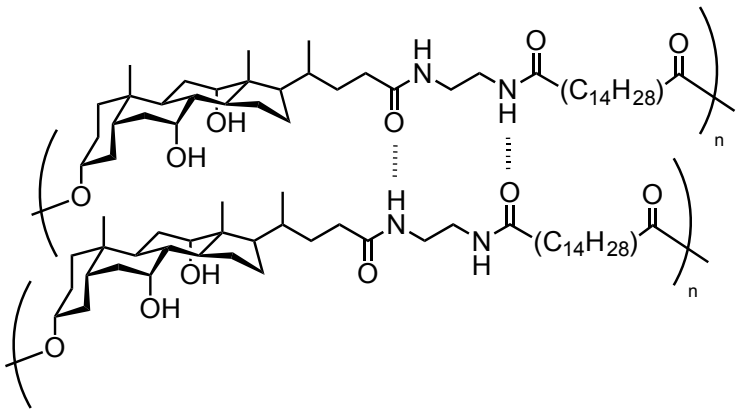
The Yamaguchi esterification is known to be efficient towards highly functionalized esters, and was previously used for the lactonization of tricolorin F.[186] In fact, this type of synthesis is said to be particularly useful for mild and rapid macrocyclization reactions, in order to overcome the entropy factor that favors oligomerization.[192] This reaction takes advantage of 4-dimethylaminopyridine's (DMAP) high catalytic activity for the transfer of acyl groups.

The work was first performed on a cholic acid moiety possessing a terminal amine (Scheme A1.1.1), since this type of functionalization could potentially offer strong mechanical properties. However, solubility issues were encountered and limited the probability of obtaining the cyclic product. The same difficulty was previously encountered when performing ring-closing metathesis with Grubbs first generation catalyst.



Scheme A1.1.1 Yamaguchi lactonization/amidation reaction conditions.

If monomers containing amide groups were produced, the resulting polymers would have stronger thermo-mechanical properties than their ester counterpart. Indeed, hydrogen bonds may be formed between polymer chains as shown in Scheme A1.1.2.

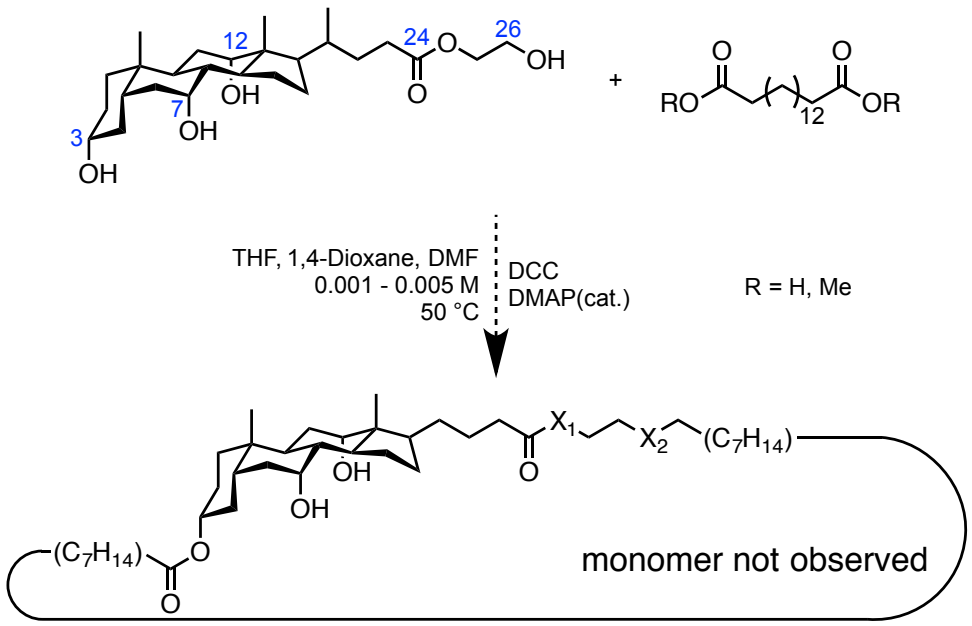


Scheme A1.1.2 Hydrogen bonding between polymer chains possessing amide linkages.

Hence, the nitrogen atoms were replaced by oxygen atoms, but the entropic barrier still remained, and no cyclic product was observed, even after trying many reaction conditions combinations.

A1.2 Steglich Esterification

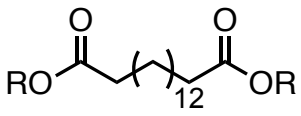
Before changing pathways towards enzymatic catalysis, Steglich esterification was tested for the lactonization of cholic acid moieties with TA (Scheme A1.2.1). As done for Yamaguchi esterification, several combinations of reaction conditions were tested. Lactonization could not be obtained; the only reactivity observed was at position 26, the only primary alcohol on the CAEG molecule. An alternative to this one-step strategy is a two-step lactonization. However, oligomerization is a possible side-reaction to consider.



Scheme A1.2.1 Steglich esterification of CAEG with TA.

A1.3 Activation of the acyl donor

In order to make the acyl donor more reactive, "good" leaving groups were introduced at both ends of TA (Scheme A1.3.1). Unfortunately, these attempts also failed, and this is when enzymatic esterification was seriously considered.



R = Me, NHS, Cl

Scheme A1.3.1 Variety of leaving groups introduced on TA.

Appendix 2 – Supporting Figures and Information

A2.1 Mass Spectrometry

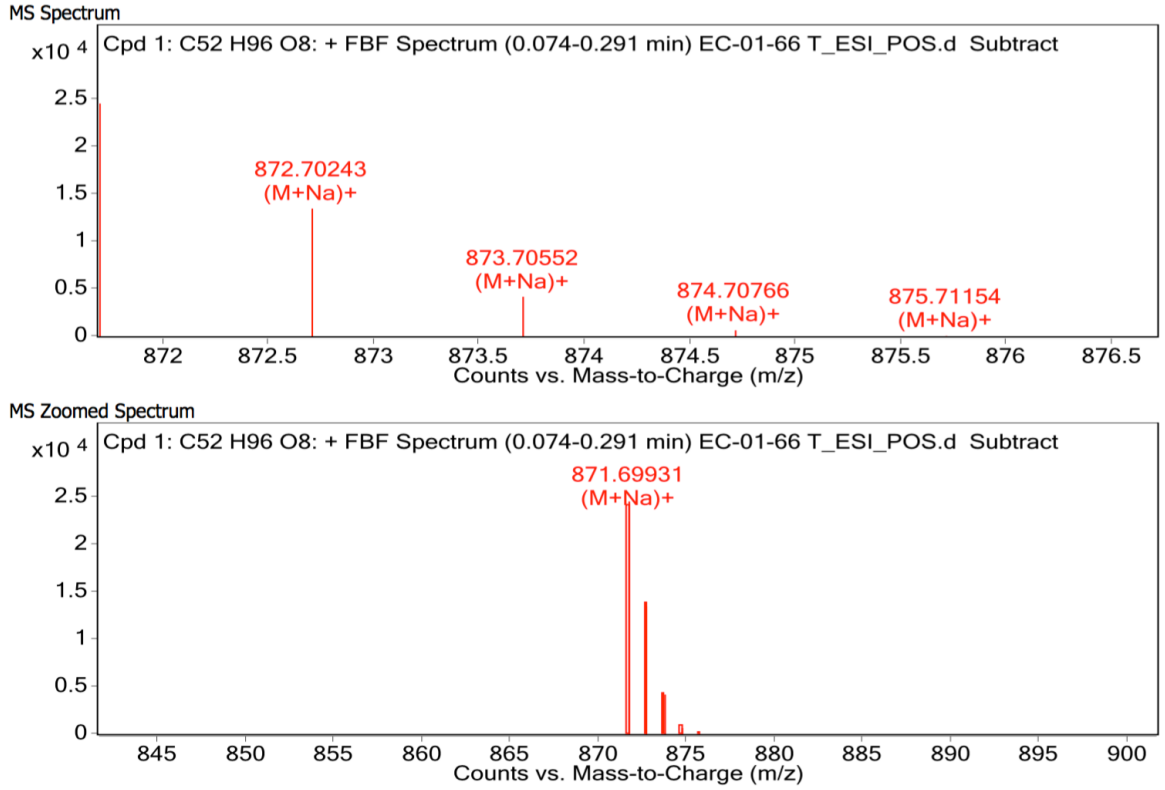


Figure A2.1.1 ESI-MS of monomer **3**.

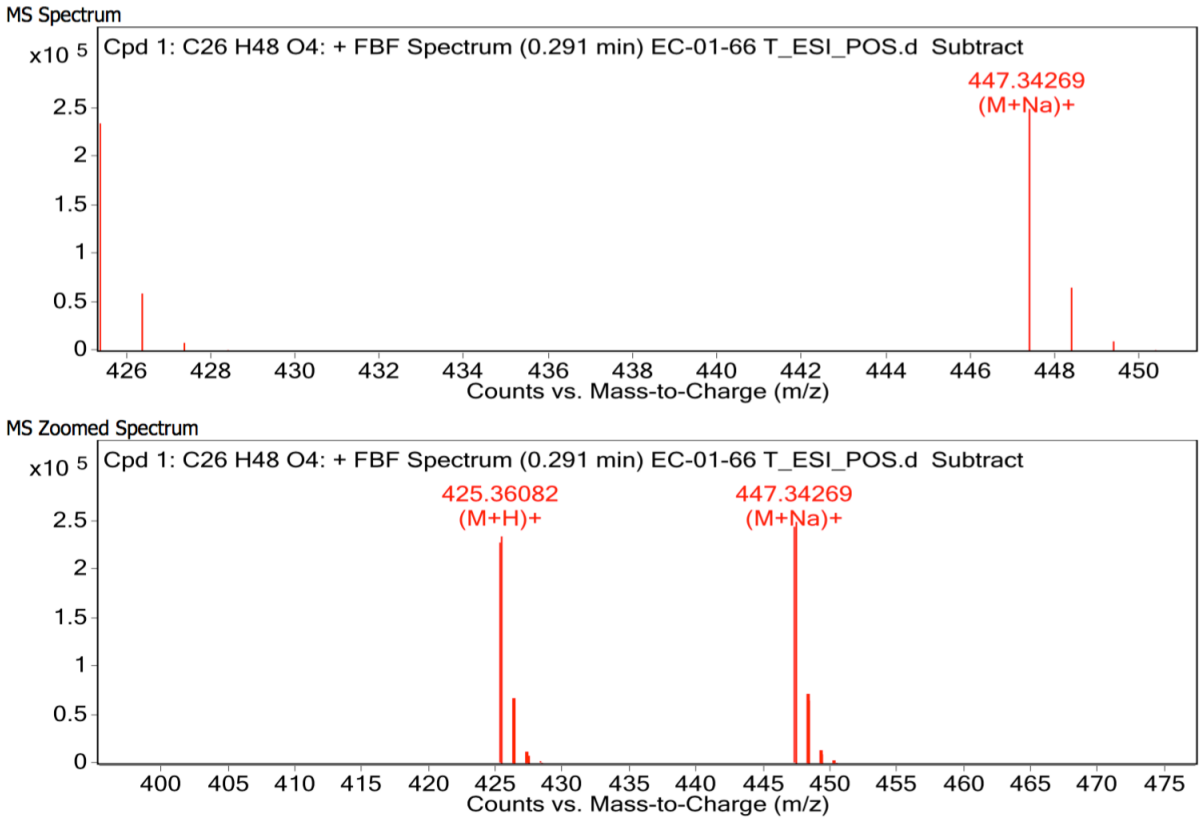


Figure A2.1.2 ESI-MS of monomer **4**.

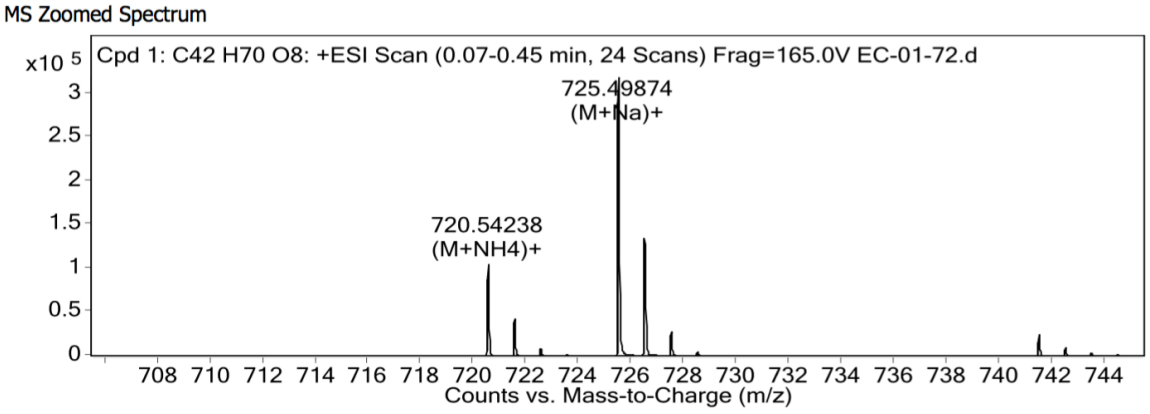


Figure A2.1.3 ESI-MS of CAEG cyclic monomer **7**.

A2.2 Thermogravimetric Analysis

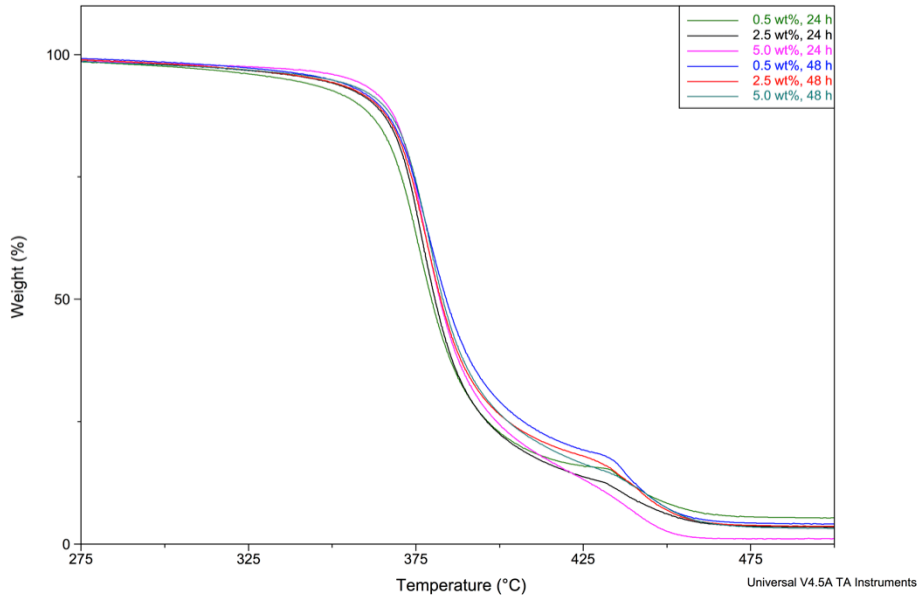


Figure A2.2.1 Overlay of TGA curves of polymer **5**, with different reaction times and enzyme amounts.

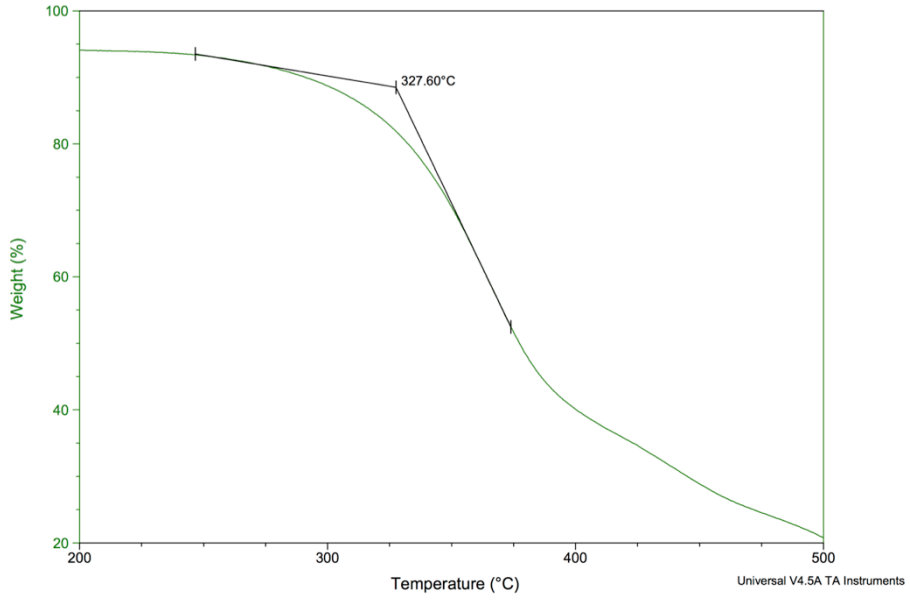


Figure A2.2.2 Monomer **7** TGA curve with T_d.

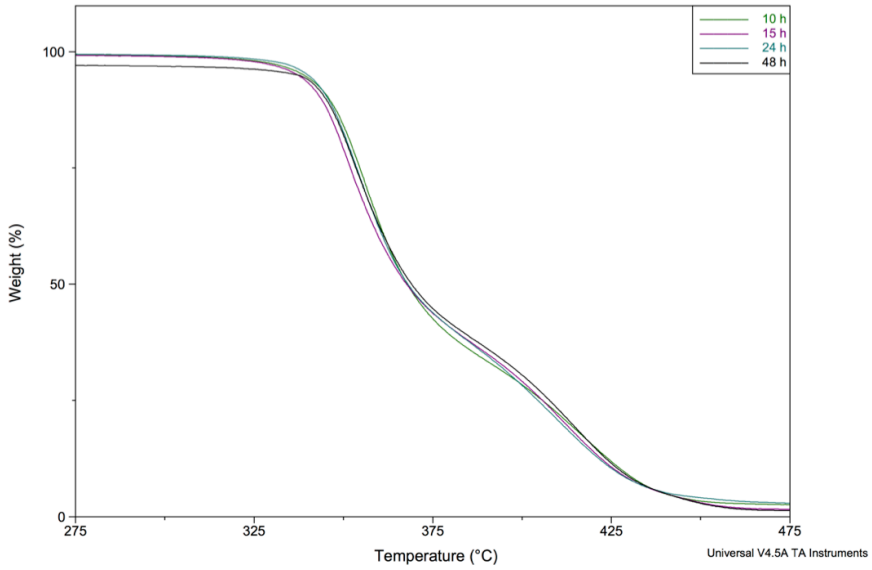


Figure A2.2.3 Overlay of TGA curves of polymer **8**, with different reaction times.

A2.3 Differential scanning calorimetry

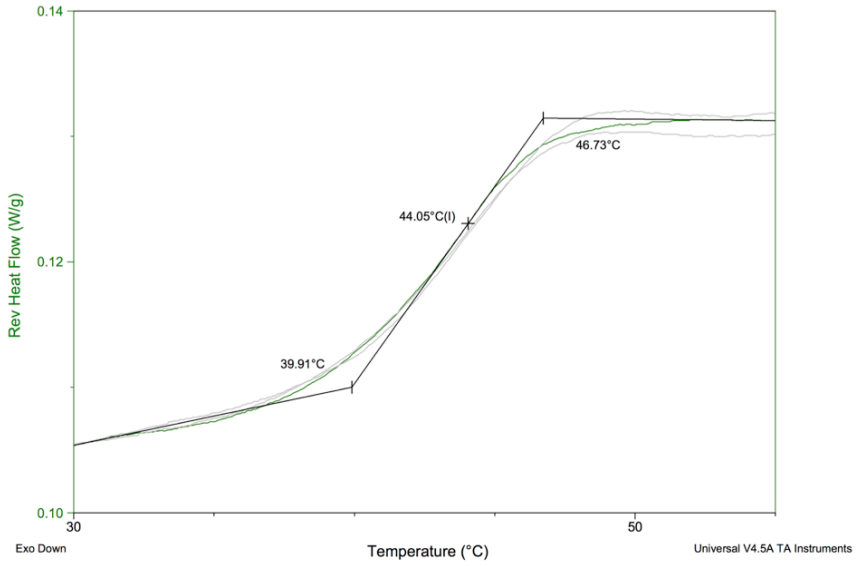


Figure A2.3.1 DSC curve showing the glass transition temperature of monomer **7**.

A2.4 Pictures of polymer 8

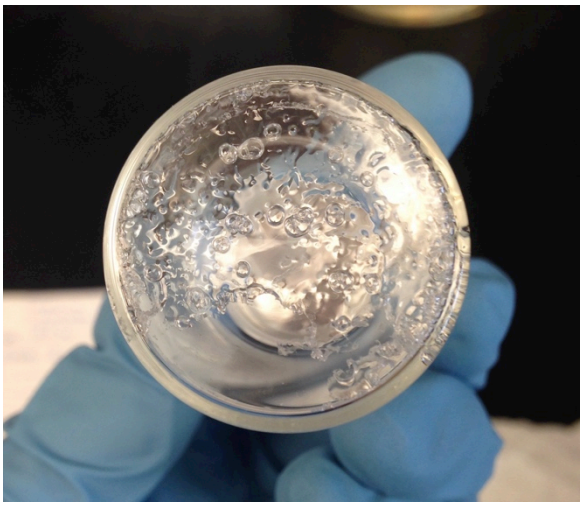


Figure A2.4.1 Bottom of vial containing a thin film of polymer **8**, showing its colorless nature.

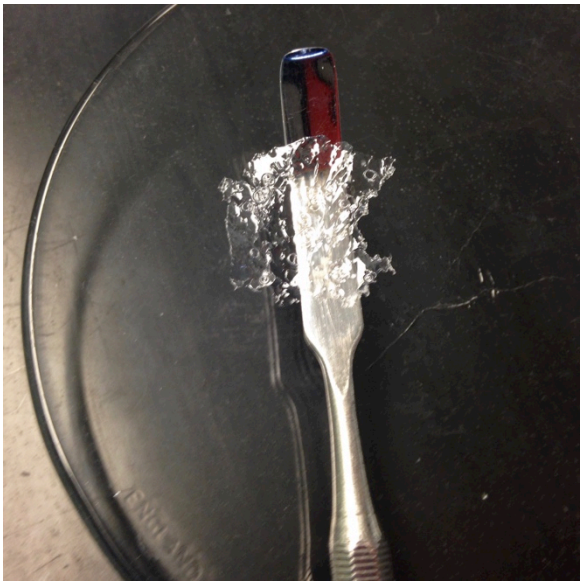


Figure A2.4.2 Thin film of polymer **8** held on a metal spatula.