Enzymatic catalysis in the synthesis of new polymer architectures and materials

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Matthijs de Geus

Cover: *'Morgenstimmung in den Walliser Alpen'*

'Matin sur les Alpes valaisannes'

'Morning mood over the Valais Alps'

Enzymatic Catalysis in the Synthesis of New Polymer Architectures and Materials

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1

The Use of Enzymes as Catalysts in Polymer Chemistry

πολυ-μερής	*having many parts*
έν ζύμη	*in yeast*
κατα-λύειν	*to loosen*
χείν (χέω)	*to pour*

1.1. Polymers and enzymatic catalysis

Polymers

> *'A polymer is a substance composed of molecules which have long sequences of one or more species of atoms or groups of atoms linked to each other by primary, usually covalent, bonds.'* [1]

Polymers are large molecules, built up in a repetition of many identical, similar, or complementary subunits, which are called monomers. Polymers can originate from natural sources or can be produced synthetically by chemical approaches. 'Natural' polymers include starch, rubber, silk and DNA, and all of these are produced by biochemical multi/step processes in a living cell. Synthetic polymers have been first commercialized in 1910 (Bakelite). Since then, the library of synthetic polymers has been drastically extended, and polymers have obtained a dominating position in our daily life by the implementation in all kinds of materials, such as clothing, building materials, coatings, adhesives, and many other applications. Annually, almost 150 million tons of plastics are produced, and still this is the fastest growing market among structural materials.

Polymers can be classified in three main clusters: thermosets, thermoplastics and elastomers. Thermoset materials usually consist of small polymer molecules in liquid or powder form, and are designed to be cured into their final form or used as adhesives. A thermoset material cannot be melted and re-shaped after it has been cured, and it is normally more brittle than a thermoplastics material. Coatings and adhesives are two examples of thermoset materials. Thermoplastic materials are linear or branched polymers which can be melted upon the application of heat. Thermoplastic materials are often referred to as plastics, derived from the Greek word 'πλασσειν', which means 'to mold' or 'to shape'. Plastic materials can be molded and remolded into all kinds of shapes, and constitute the largest part of polymers in industry. Examples of thermoplastic materials are poly(ethylene terephtalate) (PET; soda-bottles), polystyrene (plastic cups), and poly(methyl methacrylate) (Plexiglas^{TM}). A subdivision can be made within the class of thermoplastics into amorphous and (semi-) crystalline polymer materials. Finally, elastomers are crosslinked rubbery polymers that can be elastically deformed to high extensions and can rapidly recover to their original dimensions when the applied stress is released.

Catalysis

> *'The catalytic force is reflected in the capacity that some substances have, by their mere presence and not by their own reactivity, to promote changes in otherwise stable and unreactive molecules...'*
>
> **Jöns Jakob Berzelius (1779–1848)** [2]

The principle of catalysis was applied in chemistry more and more in the first half of the nineteenth century, without realizing it. In 1835, Berzelius noticed the common factor in all previous studies and stated the definition above. This statement led to new understanding and the discovery of a variety of new processes. In the early twentieth century, catalysis was considered from a thermodynamic point of view, independently by Eyring[3], and Evans and Polanyi[4] (1935). Both papers stated that most reactions occur, if the bonds in a molecule are activated, forming a transition state. In order to overcome this activation barrier, the molecule needs any form of energy (thermal, irradiation, etc.). The role of the catalyst is to decrease the activation barrier, by allowing the reaction to proceed via another reaction path, and thus decreasing the amount of energy needed (Figure 1-1). In a non-catalyzed chemical reaction, a system has to overcome an activation barrier, $\Delta G^{\ddagger}$, whereas in a catalyzed system, this barrier is reduced to a transition state with a lower Gibbs free energy, $\Delta G_C^{\ddagger}$. Hence, non-catalyzed reactions will take place at high temperatures, whereas if the same reaction is catalyzed, it will take place at a lower temperature.

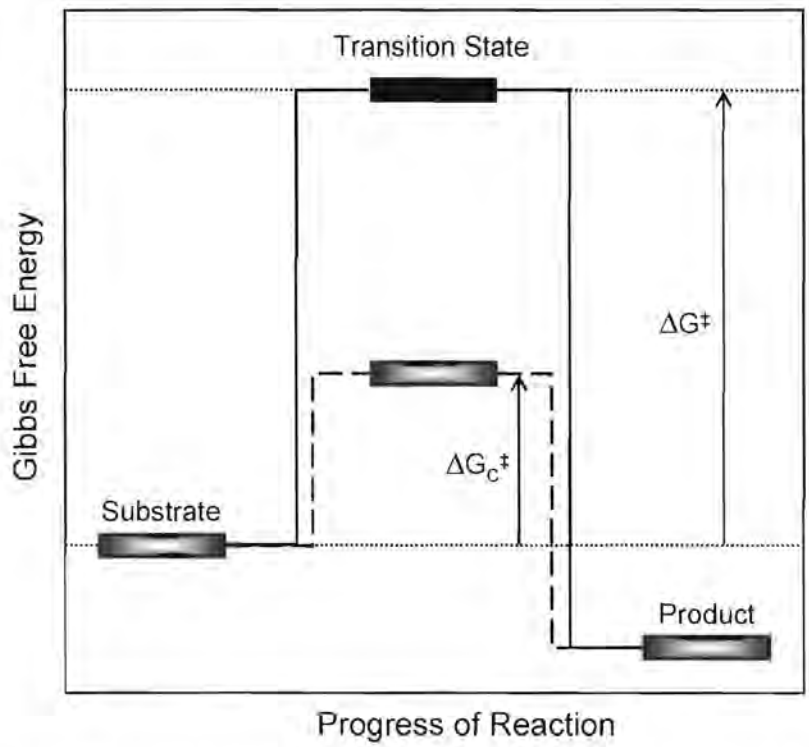

Figure 1-1: *Schematic representation of a normal chemical reaction versus a catalyzed reaction.*

Enzymes

> *'Enzymes are molecules that are complementary in structure to the activated complexes of the reactions they catalyze... ... the attraction of the enzyme for the activated complex would thus lead to a decrease in the energy of activation of the reaction and to an increase in the rate of reaction.'*
>
> **Linus Pauling (1901-1994)** [5]

Enzymes are the catalysts that Nature uses to accelerate biochemical reactions. Over millions of years, organisms have had time to adapt to the most optimal conditions under which these reactions have to be performed. In Nature, all different organisms have their own specific enzymes, as their environment can vary considerably. The power of enzymes has been known for centuries, primarily in the production of alcoholic drinks, such as beer and wine. Nevertheless, the working of enzymes was not understood at the time. In 1867, Wilhelm Friedrich Kühne suggested to combine all 'ferments' and other biocatalysts in one group called 'the enzyme', meaning 'in yeast'. Most enzymes are considered as a special class of proteins, which are natural polymers, and perform numerous functions in organisms. They are built up from amino acids, i.e. the monomers or building blocks (Figure 1-2). Although a general structural formula exists for amino acids, twenty different R-groups can be distinguished, changing the functionality of the building block.

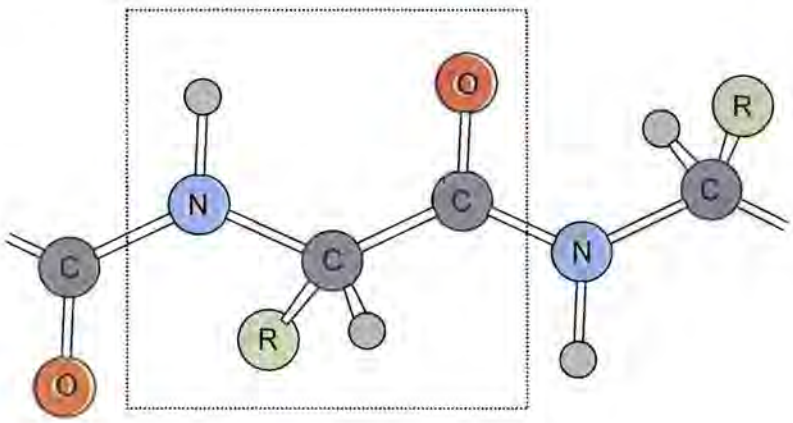

Figure 1-2: *Representation of an amino acid in a chain of amino acids, all together forming a protein.*

Most synthetic polymers are synthesized from 1-3 different monomers, while up to twenty different amino acids are available to construct enzymes (Figure 1-3). By changing the R-group, the properties of the protein can be delicately fine-tuned. When considering the macrostructure of the enzymatic polymer chain, four different levels can be distinguished. The primary structure represents the order of the amino acids. The secondary structure concerns the three-dimensional shape of the enzyme by non-covalent interactions between

neighboring amino acid residues. These interactions can be either intra- or 'inter'-molecular, resulting in different structures, α-helices and β-pleated sheets, respectively. The tertiary structure represents the conformation of the full polypeptide chain and is determined by all kinds of interactions (e.g. hydrogen bonding, electrostatic interactions, hydrophobic interactions, Van der Waals-interactions, etc.) between the different side groups of non-neighboring amino acids. This tertiary structure determines the conformation of an enzyme by the previously mentioned interactions, and hence its robustness, activity and efficiency. Finally, some proteins consist of more than only one polypeptide chain. The quaternary structure represents how several polypeptide chains are assembled together.

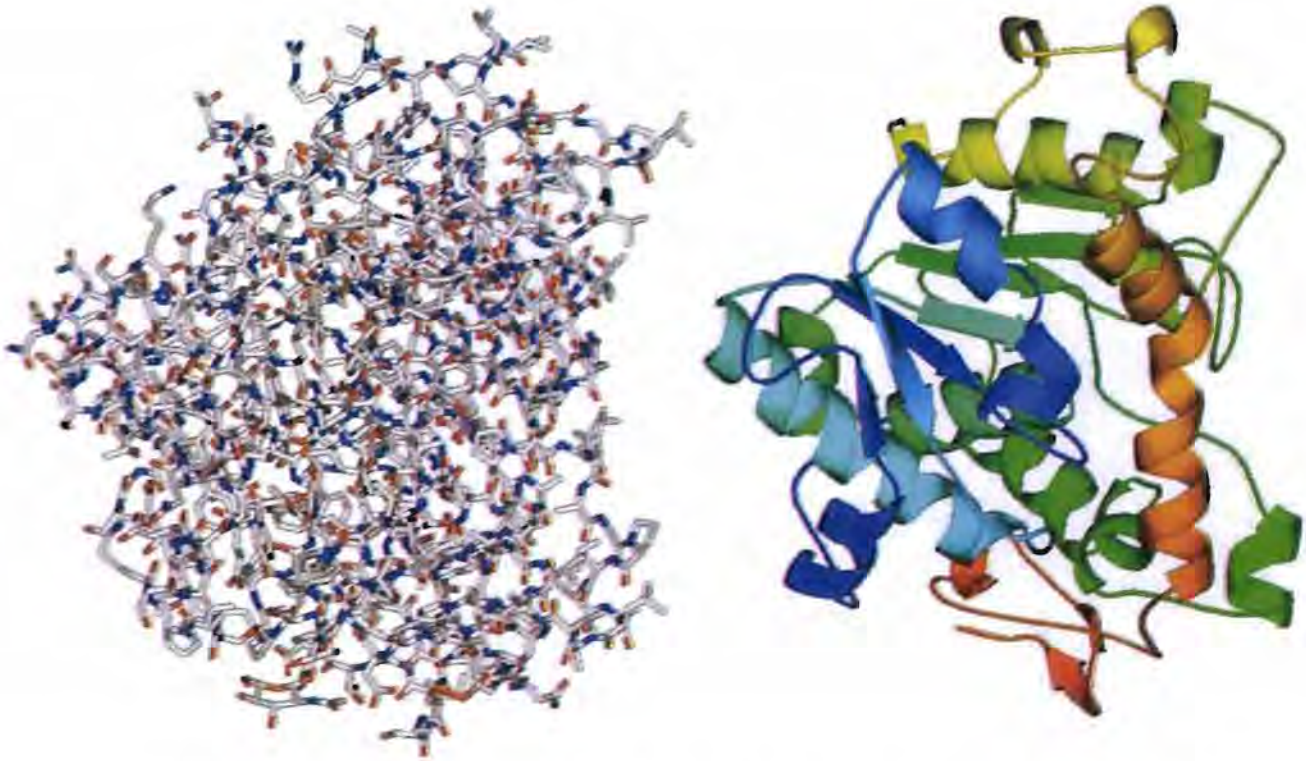

Figure 1-3: *Schematic representations of* Candida antarctica *Lipase B (CALB);* (left)*: structure of CALB visualizing the amino acid sequences;* (right)*: schematic drawing of CALB visualizing the enzyme's secondary (α-helices and β-pleated sheets) and its tertiary structure, which provides the overall conformation.*

1.2. The implementation of enzymes into polymer chemistry

Two types of enzymatic polymerization can be distinguished, *in vivo* and *in vitro*. Examples of *in vivo* enzymatic polymerizations include the formation of DNA and proteins, but also the production of spider-silk[6] and the production of poly(4-hydroxybutyrate).[7] Complex metabolisms are normally needed for the production of these polymers, which often result in very pure and well-defined material. On the contrary, enzymes can been isolated

from living organisms, such as bacteria, and applied in water or even organic media to promote specific reactions without the need of the entire cell in the organism. This enzymatic process is referred to as *in vitro* catalysis. At present, enzymes have been applied in many industrial (organic) processes in order to reduce the number of reaction steps or to introduce chirality into the end-product. In Nature, enzymes can be divided into six classes[8]:

I.	Oxido-reductases	Catalyze redox-reactions by electron transfer.
II.	Transferases	Catalyze the transfer of a functional group, for example a methyl group or a glycosyl group, from one compound (donor) to another compound (acceptor).
III.	Hydrolases	Catalyze the hydrolysis of various bonds in order to transfer functional groups to water.
IV.	Lyases	Catalyze the cleavage of C-C, C-O, C-N and other bonds by otherwise than by hydrolysis or oxidation.
V.	Isomerases	Catalyze either racemization or epimerization of chiral centres; isomerases are subdivided according to their substrates.
VI.	Ligases	Catalyze the coupling of two molecules with concomitant hydrolysis of the diphosphate-bond in ATP or a similar triphosphate.

From these six classes of enzymes, only three have been reported to catalyze or induce polymerization *in vitro*, i.e. oxido-reductases, transferases, and hydrolases. Most oxido-reductases contain low-valent metals as catalytic centre, such as iron(III) (horse radish peroxidase, HRP), copper(I) (laccase), and manganese(II), (manganese peroxidase). HRP is able to catalyze monomer activation of phenol- and aniline-derivatives. It catalyzes the decomposition of hydrogen peroxide at the expense of aromatic proton donors, allowing polymerization. In addition to polyphenols and polyanilines, HRP/hydrogen peroxide can also induce polymerization of vinyl monomers, such as styrene[9] and methyl methacrylate.[10] Transferases, such as phosphorylase and glycosyl transferase, catalyze group transfer reactions. The primary example of the potential of a transferase is the synthesis of amylose from D-glucosyl phosphate and oligomers with a minimum length of four glucosyl-residues as a primer by using potato phosphorylase.[11]

Hydrolases are the most-investigated enzymes for *in vitro* synthesis. This class includes *glycosidase*, which is used in the synthesis of polysaccharides, *protease*, which can be used for peptide bond formation, and *lipase*, which is used for the hydrolysis of fatty esters in nature. The latter is particularly interesting for polymer synthesis. Lipases are known to catalyze reactions in organic media, since they are active on the water-fat interface in cells. Moreover, lipases do not require any cocatalyst. Lipase can be used for polycondensation and

polytransesterification reactions, ring-opening polymerizations, and polymer modifications reactions. This thesis only describes the results of enzymatic ring-opening polymerization by lipase. Hence, the use of the word enzyme refers to lipase only.

The application of enzymes in polymer chemistry has many advantages: polymerizations can be performed under mild conditions with regard to pressure, temperature and pH, which makes enzymatic reactions very energy efficient. Enzymes can be highly selective: chemo-, regio-, and enantioselectivity can all be enzymatically induced, opening a novel direction towards precision polymer synthesis. Enzymes are considered to be 'green', non-toxic catalysts, which can meet the increasing demands regarding commercial, ecological and biomedical requirements. The application of enzymes is not limited to their original environment or natural role: lipases can catalyze hydrolysis, but can also catalyze the opposite reaction, i.e. condensation.

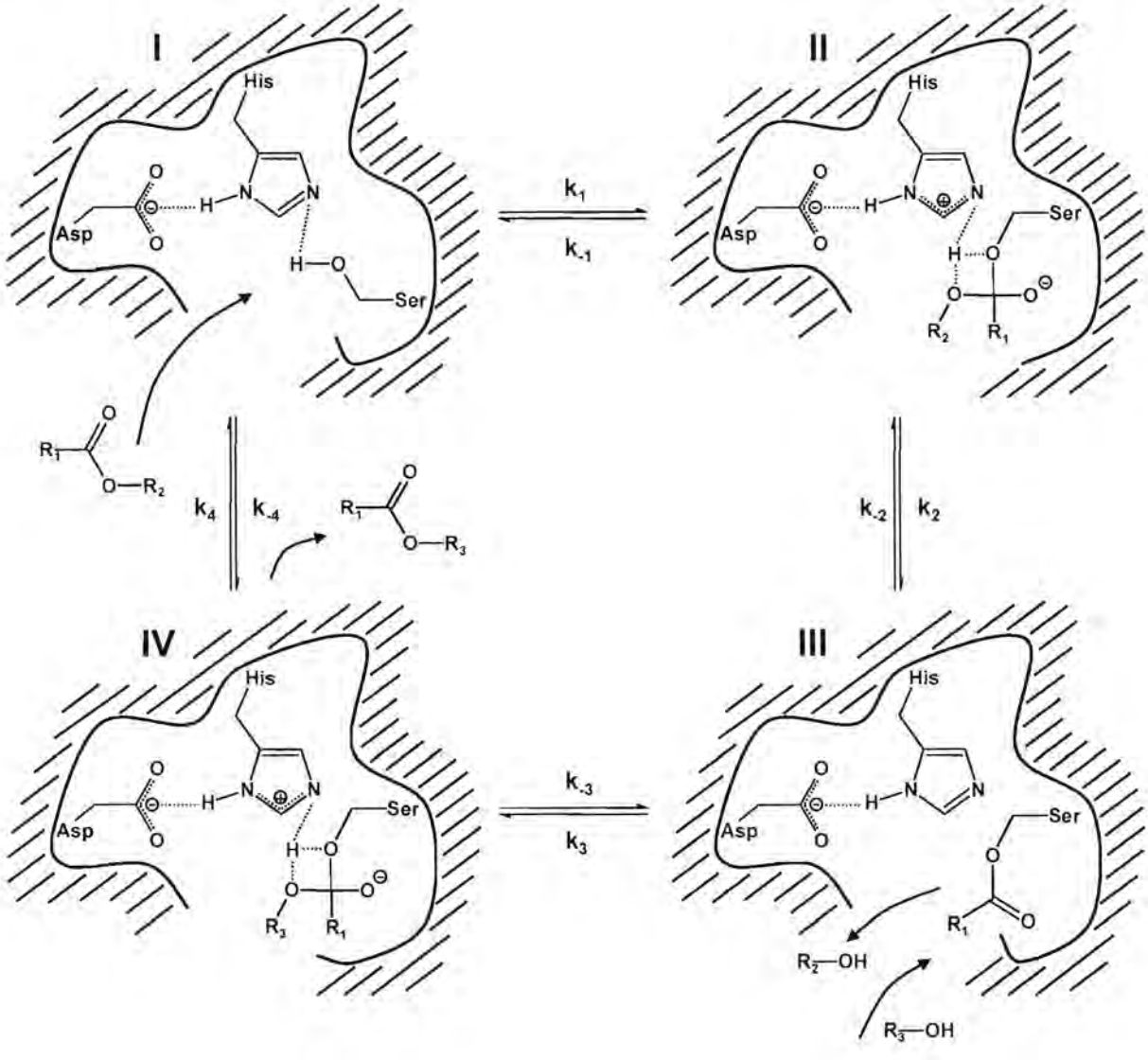

Figure 1-4: *Proposed mechanism for enzymatic transesterification.*

1.3. Enzymatic ring-opening polymerization

Principle of e-ROP

Figure 1-4 shows the mechanism of enzymatic transesterification from which the mechanism of enzymatic ring-opening polymerization (e-ROP) is derived.[12, 13] The active site of a lipase is generally formed by a catalytic triad consisting of serine, histidine and aspartate, which are electronically stabilized. An ester functions as substrate molecule and undergoes a nucleophilic attack from the primary alcohol group of serine in the enzyme's active site (I). Via the enzyme intermediate species (II) the original alkoxy-group will be released, forming the so-called enzyme-activated monomer species, EAM (III). Subsequently, a nucleophile, e.g. a primary alcohol (R_3-OH) can attack this EAM-species, and via the new intermediate species (IV) the final product is released, thereby regenerating the enzyme.

Enzymatic ROP is a form of transesterification. Here, cyclic esters (lactones) are used as substrate and are opened by the enzyme. The nucleophile, R_3-OH, that is necessary to regenerate the enzyme and create the (ring-opened) product, can be considered as the initiator of the polymerization. This initiator can be water or any other nucleophile (such as alcohols, amines and thiols).[12, 14] The ring-opened product that is formed after one catalytic cycle consists of a hydroxyl-moiety on one side and the initiator-functionality on the other. In a succeeding catalytic cycle, new substrate is activated by the enzyme. Propagation occurs by a nucleophilic attack of the hydroxyl-moiety of the ring-opened product from the previous cycle. In addition to monomer activation, also the ester bond of the polymer can be activated, forming the enzyme-activated polymer chain (EAPC)[15]. In this thesis, transesterification reactions will refer to this polymer activation. When monomer is activated, the term propagation will be used.

Michaelis-Menten kinetics

Leonor Michaelis and Maud Menten proposed a simple model to describe enzymatically catalyzed reactions, which accounts for the kinetic properties of many enzymes:[16]

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P \qquad (1.1)$$

Enzyme (E) and substrate (S) form an intermediate complex (ES), after which the product (P) will be released from the enzyme. From equation 1.1 the rate of the catalytic reaction can be given by:

$$V = k_3[ES] \qquad (1.2)$$

If steady-state conditions are valid, the rate of formation and decomposition of the enzyme activated species, ES, are equal, thus:

$$[ES] = \frac{[E][S]}{(k_2 + k_3)/k_1} \tag{1.3}$$

Now, the ratio of reaction rate constants can be written as K_M:

$$K_M = \frac{k_2 + k_3}{k_1} \tag{1.4}$$

This parameter K_M depicts the ratio of rate constants of the breakdown versus the formation of the enzyme activated species, ES. A high K_M reflects faster breakdown than formation of ES, whereas a small K_M reflects faster formation than breakdown of ES. The total concentration of active sites, $[E_T]$ has to be divided over free sites, [E], and occupied sites (enzyme-activated substrate), [ES]. When this is substituted and combined with equations 1.2-1.4, the equation 1.5 is obtained:

$$[ES] = [E_T]\frac{[S]}{[S] + K_M} \tag{1.5}$$

The maximal rate of catalysis, V_{max}, is attained when all the catalytic sites on the enzyme are occupied with substrate, i.e. when [S] is much greater than K_M. Thus,

$$V = V_{max}\frac{[S]}{[S] + K_M} \tag{1.6}$$

which is generally referred to as the Michaelis-Menten equation. In enzymatic reactions, V_{max} and K_M can be determined for any specific substrate. K_M reflects the affinity of one specific enzyme for one specific substrate, and V_{max} reflects the maximum rate of catalytic reaction, i.e. when all the catalytic sites on the enzyme are occupied with substrate. It must be noted that this relationship has been derived for a single-substrate single-product reaction. Theoretically, enzymes often follow more advanced and complex kinetics, but, in practice many enzymes seem to obey Michaelis-Menten kinetics. Therefore, traditionally, the kinetics of many enzymatic reactions are calculated according to equation 1.6, referring to a substrate-specific K_M and V_{max}.

1.4. Current status of enzymatic ring-opening polymerization

The first in-vitro example of e-ROP was published in 1993, when Knani and coworkers[17] and Kobayashi and coworkers[18] independently reported on ring-opening polymerization of ε-

caprolactone (ε-CL) catalyzed by lipase. The molecular weight of the obtained PCL was low (M_n < 2000 g/mol) and polydispersity typically exceeded 5. Since then, several lipases were screened to perform e-ROP, transesterification, and polycondensations.

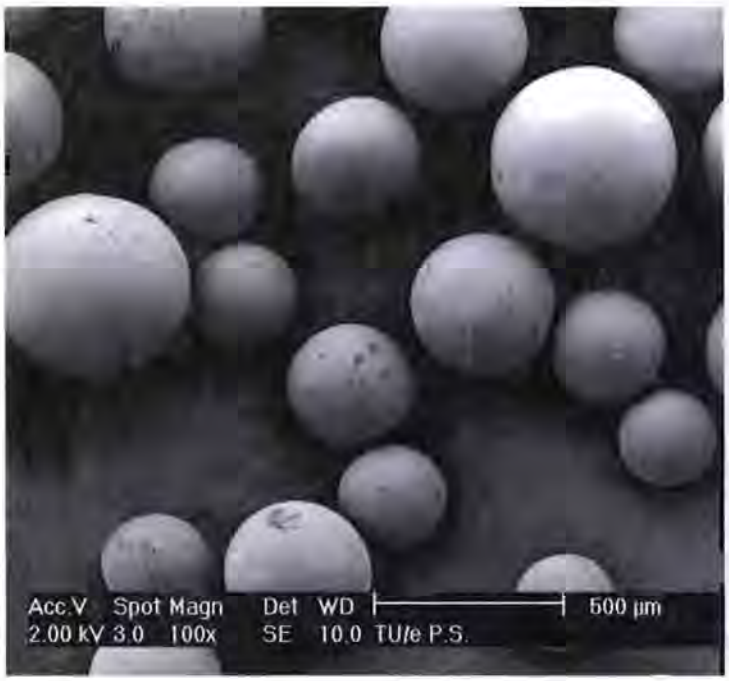

Figure 1-5: *Scanning electron microscopy (SEM) image of Novozym 435,* Candida antarctica *Lipase B (CALB) on a macroporous crosslinked acrylic resin.*

An extensive summary of the lipases investigated in enzymatic polymerizations was published by Kobayashi and Gross in 2001 [11, 19], and updated by Albertsson in 2005[20]. Over the last ten years, most groups have used Novozym 435 for e-ROP. Novozym 435 is a commercially available *Candida antarctica* Lipase B, (CALB), immobilized on a macroporous crosslinked resin of poly(methyl methacrylate) (Lewatit VP OC 1600, Bayer).[21] Novozym 435 is produced by Novozymes A/S and is probably the most applied enzyme in organic chemistry.[22] It is a highly versatile catalyst with activity towards a great variety of different substrates. The immobilized enzyme is thermostable and a robust catalyst with activity in various organic solvents (Figure 1-5). Although Novozym 435 is used in industrial processes as a catalyst in the production of simple esters and amides and in the regioselective synthesis of carbohydrate monoesters, it is primarily used as a highly enantioselective catalyst in the synthesis of optically active alcohols, amines and carboxylic acids.[23] Over the past years, Novozym 435 has also been applied in polymer chemistry in the synthesis of monomers, polymers, and modification as well as degradation of polymers.[11, 24, 25]

As lipases can catalyze polycondensations, polytransesterification reactions, and ring-opening polymerizations, a large group of monomers can be applied. For polycondensations, diols and diacids are used (AA/BB), although also hydroxy-acids (AB) can be applied as

monomers in this type of polymerizations. In polytransesterification reactions, normally the diacid derivates are applied as monomers. Also anhydrides can be used for the enzymatic synthesis of polyesters. Finally, e-ROP needs cyclic monomers, hence, lactones are an appropriate group of monomers. e-ROP of non-substituted lactones has been reported for almost all ring sizes, from 4-membered to 17-membered lactones.[11, 26] Additionally, substituted lactones, cyclic carbonates, and cyclic diesters have been polymerized.[11] A very obvious advantage of enzymes with respect to more classical, often metal-based catalyst, is the formation of chiral polymers from substituted lactones, due to the preference of the enzyme for one of the enantiomers.

1.5. Outline and aim of the thesis

Over the last decade, studies on enzymatic ring-opening polymerization have resulted in many examples of enzymatic polymerization which opened a route to a novel, biocatalytic, and cleaner process for the synthesis of polymeric materials. So far, research has predominantly been focused on how to use the enzyme's selectivity to modify polymers selectively, which cannot be easily achieved via chemical routes.[27-29] Moreover, well-established polymers have been enzymatically synthesized for biomedical applications, without the use of a metal catalyst.[12, 30-33] However, only few reports have been published on new materials, which are not readily accessible via traditional polymerization techniques.[13, 34-36] The aim of the work described in this thesis is to prove the compatibility of enzymes and e-ROP with other polymerization techniques and the corresponding catalysts. In addition, enzymes are used to synthesize new polymers, which cannot easily be obtained with high molecular weight via chemical routes.

Chapter 2 focuses on the mechanism and kinetics of e-ROP to answer the question if e-ROP can be considered as a controlled polymerization technique. A new kinetic expression for e-ROP will be evaluated. In addition, several parameters that influence kinetics and enzyme activity are discussed. It was observed that parameters such as water concentration and monomer concentration have a dramatic influence on the obtained polymer structure. In Chapter 3, these parameters have been varied in order to show their effect on the kinetics of the reaction and the structure of the obtained polymer species. A relatively unknown characterization technique, i.e. liquid chromatography under critical conditions (LCCC), has been utilized to quantify the presence of the obtained polymer species. From these results new insights were obtained into the process of e-ROP. The results and insights obtained in Chapters 2 and 3 have been used to synthesize block copolymers via a chemo-enzymatic approach by applying e-ROP and a controlled radical polymerization technique, atom transfer radical polymerization (ATRP) from a bifunctional initiator. In Chapter 4, both indirect and

direct synthesis routes are presented to prove the compatibility of enzymes towards other (metal-) catalysts and reactants.

In Chapter 5, the enzymatic synthesis of high molecular weight material poly(ω-pentadecalactone), PPDL, is described in detail, discussing both the route to obtain high molecular weight polyester and the approaches and challenges to up-scale this synthesis. In addition, material properties of PPDL and different applications of this polymer are evaluated. Finally, Chapter 6 looks into the possibilities of enzymatic polymerization for the synthesis of new materials, using the approaches that are described in the previous chapters. The results presented throughout this thesis are brought into perspective. Beside the goals that have been achieved, expected future developments are discussed in this chapter as well.

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2

Enzymatic Ring-Opening Polymerization as Synthetic Tool in Polymer Chemistry

Abstract

The mechanism and kinetics of enzymatic ring-opening polymerization (e-ROP) are discussed in order to answer the question if e-ROP is a controlled polymerization technique. Additionally, practical requirements are investigated, in an attempt to provide information on how to limit the formation of side-products. This information will allow us to obtain insight into the order of control over the polymerization.

2.1. Introduction

The last twenty years, the synthesis of controlled structures and complex architectures has become an important field in polymer research.[1-3] This has resulted in the development and application of several controlled radical polymerization techniques such as atom transfer radical polymerization (ATRP)[4-6], nitroxide-mediated living polymerization (NMP)[7-9], and reversible addition-fragmentation chain transfer polymerization (RAFT).[10, 11] Traditional polymerization techniques, such as ring-opening polymerization, have also known revived interest, especially in research towards novel (organo-)catalysts that show increased control over end-product, as well as stereo- and enantioselectivity.[12-15] Typically, these techniques have not been implemented on an industrial scale, but have so far only been applied for small scale synthetic purposes. On the contrary, typical polymerization techniques that are applied in industry, e.g. polycondensation and free radical polymerization, lack control over the reaction and the obtained polymers. The polymers are produced on a large scale, and their specific applications do not require any well-defined structures or complex architectures. Generally, polydispersities of the obtained polymers are high (>1.5) and selectivity is only statistically governed.

In enzymatic ring-opening polymerization (e-ROP), ε-caprolactone (ε-CL) is by far the most studied monomer in literature, as it is easily available and the obtained polymer is easy to characterize. Several articles have been published on e-ROP of ε-CL, showing that molecular weights between 2 000-20 000 g/mol are readily obtained at elevated temperatures (60-90 °C) in only a few hours, using Novozym 435 in various solvents.[16-18] Polydispersities typically range from 1.4-3.2 and the extent of control obtained over the polymerization is claimed to be from dramatic to perfect. Although the formation of cyclic polymer by intramolecular transesterification has been reported[19, 20], many researchers do not to take its formation into account when discussing the degree of end-functionalization.[21-23] It has been reported that temperature, water, and the solvent play an important role with respect to the final polymer product.[18, 24-26] The variation of these conditions have been extensively studied and discussed in literature, resulting in several statements concerning the control over the polymerization: e-ROP has sometimes been classified as a controlled, and even living polymerization technique, as the monomer consumption seems to obey first-order kinetics.[23, 27, 28] As e-ROP is a relatively new polymerization technique, the following question arises (Figure 2-1): 'Could e-ROP compete with existing controlled polymerization techniques, and hence be regarded as a controlled polymerization technique?'

In this chapter, we try to answer this question by carefully studying the kinetics of the e-ROP of ε-CL. The obtained polymers are analyzed with MALDI-ToF-MS, SEC and ^{1}H-NMR spectroscopy. Based on the results of these analyses, requirements in the conditions of e-ROP

will be discussed, i.e. the role of water and the formation of cyclic polymers, which are crucial when aiming for control over the polymer end-group functionality.

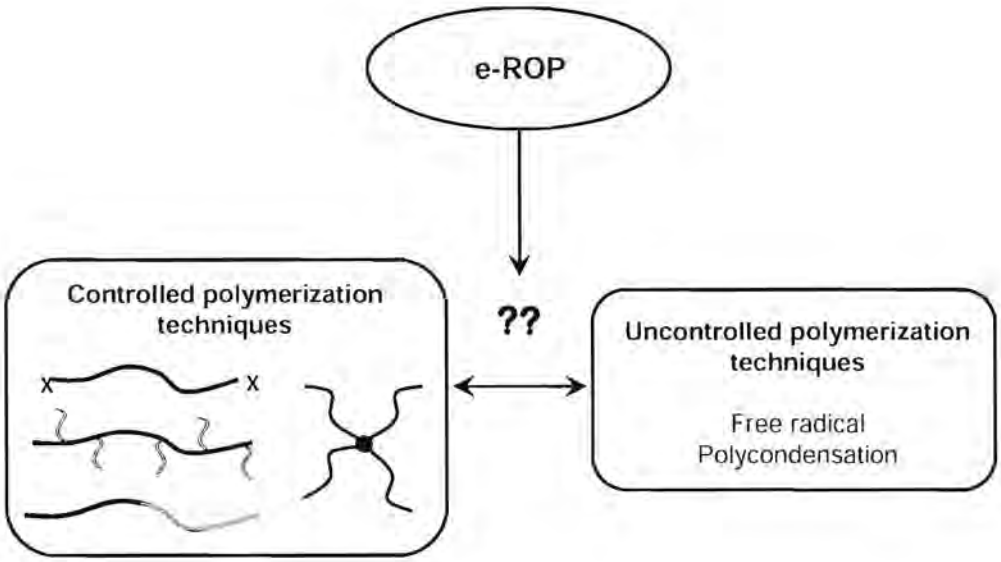

Figure 2-1: *Distinction between synthetic, controlled polymerization techniques and typical, industrially applied, uncontrolled polymerization techniques: Where does e-ROP fit in this picture?*

2.2. Propagation

It is commonly accepted that the active site of lipase consists of a triad of amino acids, serine, histidine and aspartate, and that lipase-catalyzed reactions proceed via an acyl-enzyme intermediate complex (EAM). The formation of this EAM is assumed to be the rate limiting step of the catalytic cycle.[29] In 1995, Kobayashi and Gross proposed a mechanism for enzymatic ring-opening polymerization based on this information[30, 31], which was discussed in Section 1-3. Since then, this so-called 'activated monomer mechanism' has been the standard mechanism to visualize the different fundamental steps in e-ROP.[32] In addition, a polymer chain can be end-functionalized with a specific group by using a nucleophile bearing this functionality.[21] It is believed that this nucleophile can react with the activated monomer species (EAM), thereby building its functionality into the polymer. Apart from this desired functionalized polymer chain, other polymer species, such as carboxylic acid end-functionalized polymer species and cyclic polymer species are normally obtained in e-ROP of lactones. In the following sections the formation of these species will be elaborated on, including methods to minimize their formation.

The rate of polymerization in e-ROP normally obeys Michaelis-Menten kinetics (MM)[33], which was introduced in section 1-3 (equations 1.1-1.6). However, a new expression has been derived by Van As[34], which describes the propagation reaction more accurately. MM-kinetics

are based on the assumption that the formation of product from the enzyme-substrate complex (ES) is the rate-determining step of the reaction (equation 1.1). In practice, this is an oversimplification, as more steps are involved in one catalytic cycle. Figure 2-2 depicts the suggestion by Van As, in which all fundamental steps in the catalytic cycle are taken into account. It was reported that the formation of the enzyme-substrate complex is the rate-determining step of the reaction[35], which is another indication that standard MM-kinetics are not completely applicable.

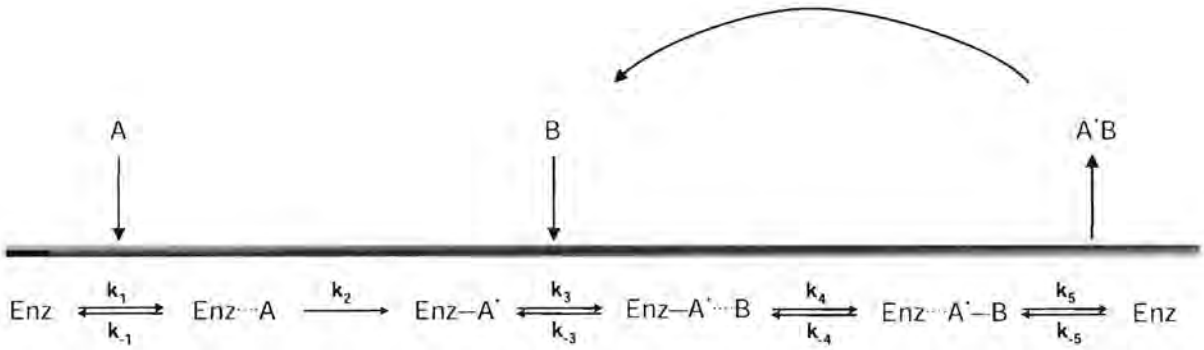

Figure 2-2: *Cleland-plot for enzymatic polymerization, based on the mechanism in Figure 1-4, and the assumption that the activation of monomer A is the rate-determining step of the reaction; B represents the nucleophile, and A˙B the ring-opened product.*

The first step in Figure 2-2 is the adsorption of monomer A by the enzyme (k_1), followed by the activation of monomer A by the enzyme (k_2). Nucleophile B will adsorb onto the formed enzyme-activated monomer species, Enz—A*, and will perform a nucleophilic attack on the activated monomer species. The last step involves the regeneration of the enzyme and the release of product A*B. The latter is also a very important step in the catalytic cycle, as the ester bond of the polymer can be (re-)activated by the enzyme, forming enzyme-activated polymer species (EAPC), which is referred to as transesterification in literature.[36] A recent article from Van der Mee et al. has reported the behavior of lactones with different sizes in e-ROP.[45] It was concluded that the conformation of the ester bond in the monomer determines the activity of the enzyme towards the monomer. It was observed that the enzyme has a preference for the transoid conformation, which can be found in macrolides (e.g. ω-pentadecalactone) and polymers. On the other hand, ε-CL has a cisoid conformation. Hence, it must be noted that the activation of polymer chains by the enzyme is more than only a side-reaction.

In order to include the occupancy of active sites by polymeric ester bonds in a kinetic expression, Van As added a polymer-term to the MM-expression (equation 2.1) to correct for the active sites that are not involved in monomer activation.

$$V = -\frac{d[S]}{dt} = V_{max}\frac{[S]}{K_S + [S] + \frac{K_S}{K_P}[P]} \qquad (2.1)$$

In which,

V	rate of propagation	[mol/Ls]
V_{max}	apparent, maximum rate of propagation	[mol/Ls]
K_S	affinity of the enzyme for substrate, S $[(k_{-1}+k_2)/k_1]$	[M]
K_P	affinity of the enzyme for product, P $[k_5/k_{-5}]$	[M]
[S]	concentration substrate (monomer)	[M]
[P]	concentration product (polymer)	[M]

The Michaelis-Menten constant, K_M, in the original MM-expression was defined as the affinity of the enzyme for a specific substrate, $(k_{-1}+ k_2)/k_1$. Here, this constant has been replaced by two terms: the affinity constant of enzyme towards the substrate (monomer), K_S, and the affinity constant of enzyme towards the product (polymer), K_P. This affinity constant of the enzyme represents the activity of the enzyme to form the enzyme-activated species, either from substrate or from product. Due to the preference of the enzyme towards the polymer, it is assumed that the ratio of $K_S/K_P>1$. The contribution of the polymer term $K_S/K_P*[P]$ will have a significant influence during the polymerization. By integrating the substrate concentration an expression can be obtained that describes the pseudo-first order kinetics of e-ROP.

2.3. Cyclic polymer formation

As shown in Figure 2-3, cyclic structures can be formed in e-ROP, thereby lacking any end-group functionality. This was already known for polycondensation reactions in which end-to-end condensation will close linear chains into cyclic structures.[37, 38] Kricheldorf et al. stated that cyclic structures can be formed as a result of the thermodynamic or kinetic preference.[39] As enzymes activate ester bonds, it can choose between monomer and polymer depending on the affinity of the enzyme towards one or the other.[40] Hence, the polymer can be regarded as a competitive substrate towards monomer, which implies product inhibition. When product is occupying the active site, transesterification can occur when a nucleophile attacks this 'enzyme activated polymer chain' (EAPC), causing chain transfer or cycle formation (as depicted in Figure 2-3).

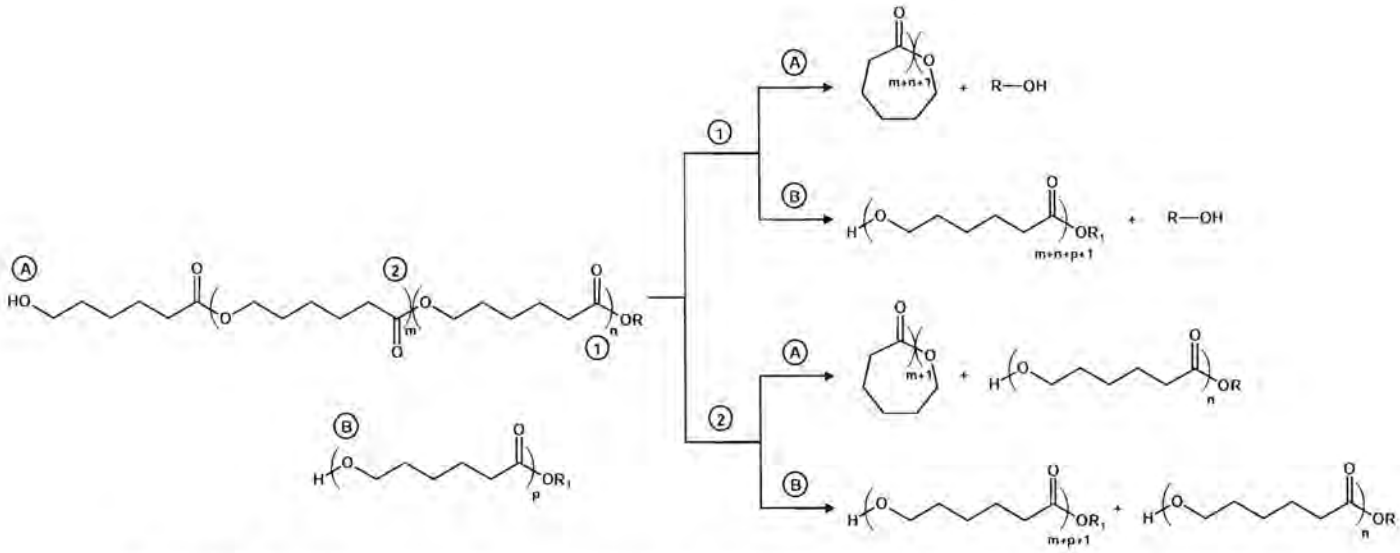

Figure 2-3: *Possible transesterification reactions in a lipase-catalyzed ring-opening polymerization; the numbers refer to the carboxylic moiety that is activated by the enzyme; the letters refer to the nucleophilic group.*

Depending on the exact place of activation, at the end of the polymer chain or along the chain, and the type of nucleophile that reacts with these EAPC-species, different polymer species can be obtained via transesterification: if a carboxylic end-group of a polymer chain is activated (**1**), and nucleophilically attacked by the hydroxyl end-group of the same chain (**A**), water or initiator will be released and a cyclic polymer chain will be formed. This type of intramolecular transesterification is called end-to-end condensation. When the activated carboxylic end-group (**1**) is attacked by the hydroxyl end-group of another polymer chain (**B**), again initiator or water is formed, but now one linear polymer chain will be formed, which can be considered as an extended chain. If a carbonyl in the polymer chain is activated (**2**), similar reactions take place. When this EAPC is attacked by the hydroxyl end-group of the same polymer (**A**), a cyclic and a linear polymer chain will be formed with lower molecular weights. When the activated carbonyl is attacked by a hydroxyl end-group of another polymer chain (**B**), two chains will be created, usually one smaller than the original chains, and one longer.

To observe the effect of concentration on the formation of cyclic polymer, e-ROP of ε-CL was performed with a bifunctional initiator, hydroxyethyl-2-bromoisobutyrate (HEBI), in different concentrations (bulk, 68, 36, and 13 w/w-% monomer in toluene) at 60 °C for 120 mins with 10 w/w-% Novozym 435 with respect to monomer. When looking at the monomer consumption as a function of time, it is observed that the polymerization is first-order towards monomer concentration (Figure 2-4A). In concentrated systems, the rate of monomer consumption is significantly lower than in dilute systems, which is probably caused by diffusion limitation (due to viscosity). The evolution of the number average molecular weight was observed to follow more or less the theoretical molecular weight (M_n(th.)) as a function

of monomer conversion (Figure 2-4B), that can be calculated from equation 2.2. Both these observations suggest that e-ROP of ε-CL has a living character with respect to control over the kinetics of the reaction.

$$M_n(\text{th.}) = \frac{[\text{Mon}]_0}{[\text{Ini}]_0} X_{\text{Mon}} M_{\text{Mon}} + M_{\text{Ini}} \qquad (2.2)$$

However, careful inspection of the evolution of polydispersity as a function of monomer conversion reveals that diluted systems eventually lead to broader molecular weight distributions (Figure 2-4C). This pattern can be attributed to the increase of transesterification reactions in diluted systems. Typical values from literature (1.5-2.0)[16] are not reached during the reaction, although precipitation of the obtained material drastically reduces the polydispersity (1.90-2.35) of these polymers, as lower molecular weight fractions are removed.

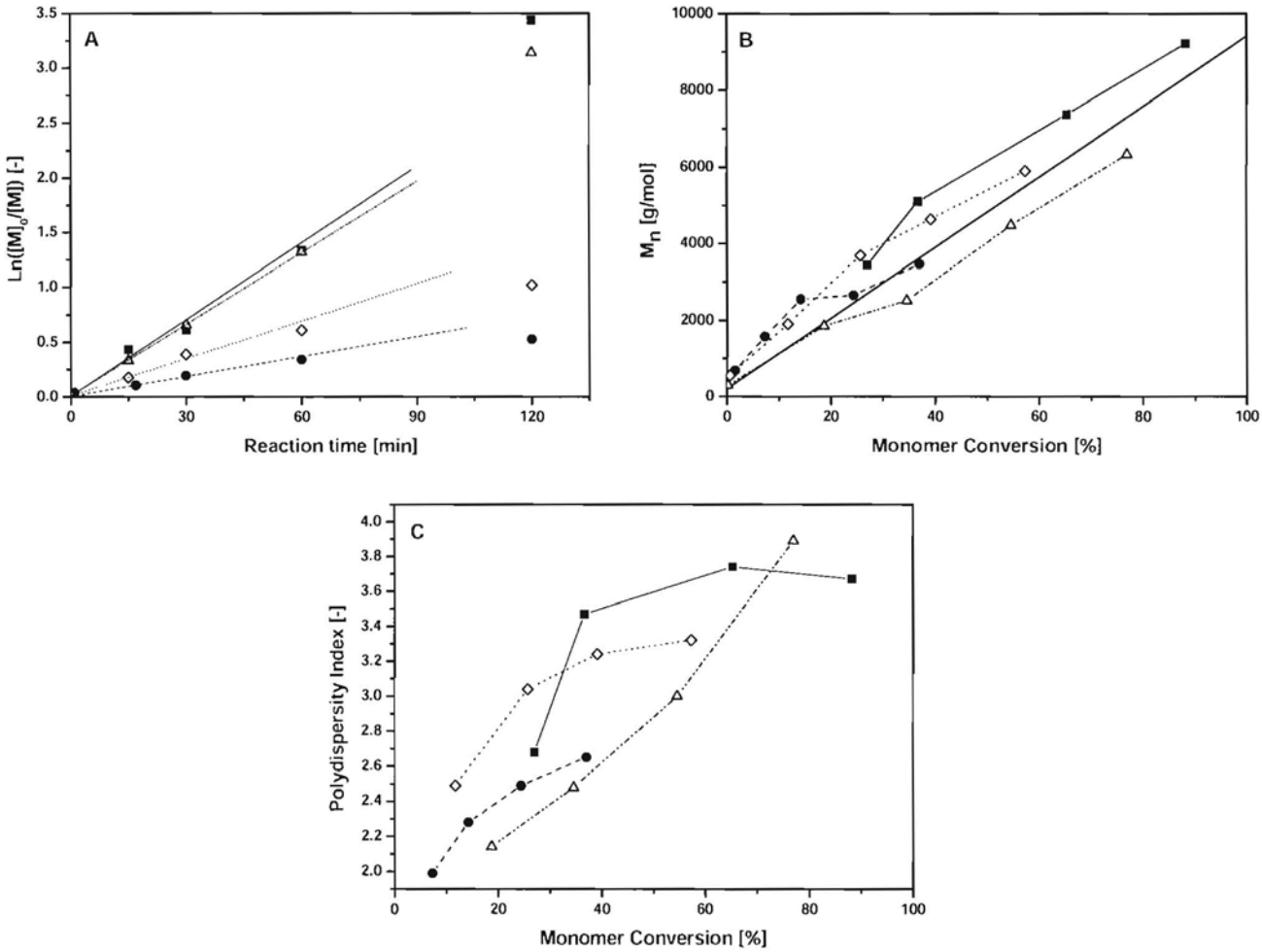

Figure 2-4: A: *Evolution of monomer consumption as a function of reaction time in e-ROP of ε-CL from HEBI with different monomer concentrations at 60 °C. Monomer consumption was determined with ^{1}H-NMR. Straight lines represent pseudo-first order kinetics.* B: *Evolution of M_n as a function of monomer conversion.* **C:** *Evolution of polydispersity as a function of ε-CL conversion. Polydispersity index was determined with SEC; ●: bulk conditions; ◊: 68 w/w-% ε-CL; ■: 37 w/w-% ε-CL; Δ: 13 w/w-% ε-CL in toluene.*

The obtained polymers were analyzed with MALDI-ToF-MS. In Figure 2-5, the MALDI-ToF-MS spectra of all four polymers are depicted, from bulk conditions to the most diluted conditions. Two distributions can be easily distinguished. At low molecular weights, a distribution of cyclic polymer structures is observed, whereas at higher molecular weights the second distribution arises. This distribution represents linear polymer chains bearing the end-group functionality introduced by the bifunctional initiator. From these spectra, it is evident that the formation of cyclic polymer is favored over linear polymer chains at lower monomer concentrations, which is in good agreement with literature[38], and is an indication that the formation of cyclic polymer structures is kinetically driven (intramolecular transesterification). Unfortunately, MALDI-ToF-MS can only be utilized as a qualitative technique, which makes it difficult to provide information on the actual amounts of cyclic or linear polymer species that are formed. In Chapter 3, this issue will be further elaborated on, by applying liquid chromatography under critical conditions (LCCC).

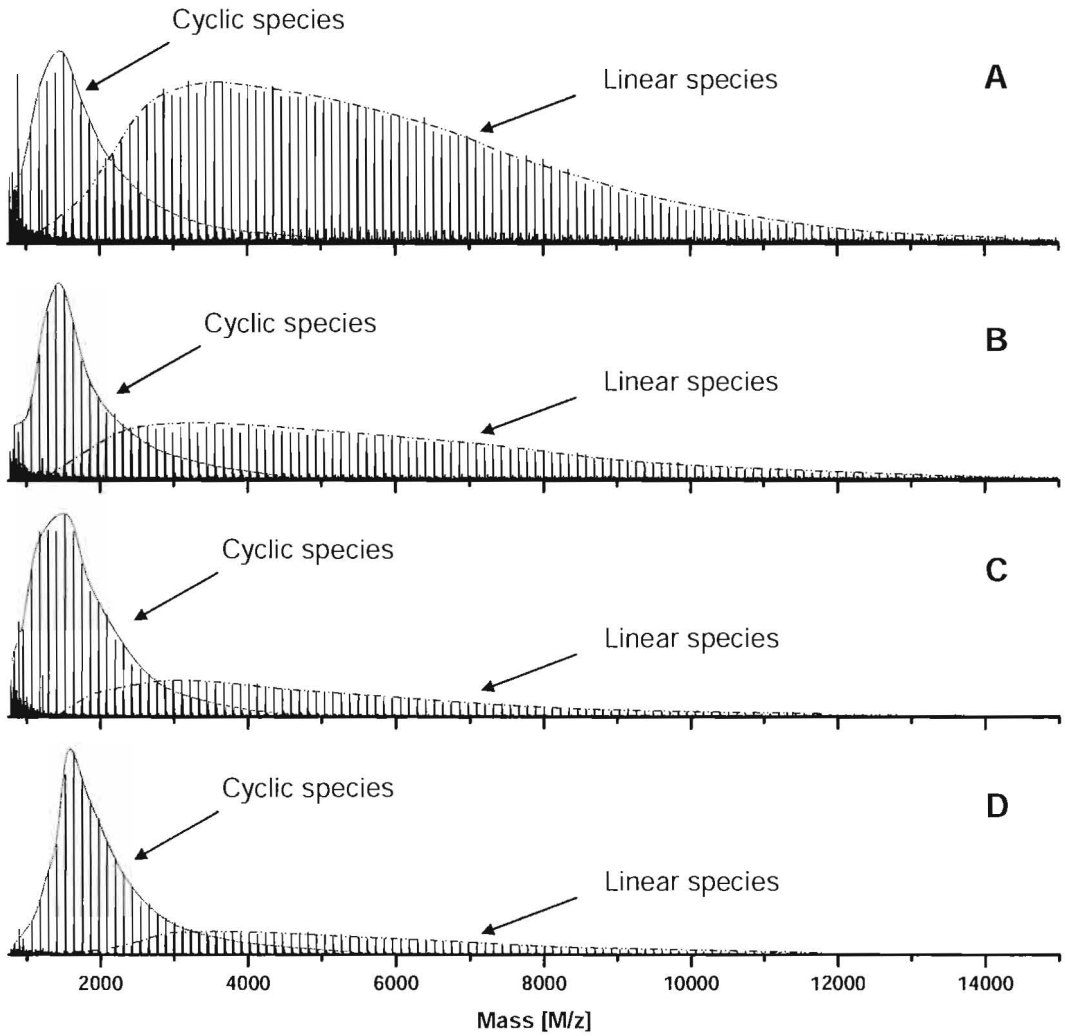

Figure 2-5: *MALDI-ToF-MS traces of PCL obtained from e-ROP with different monomer concentrations.* ***A:*** *bulk,* ***B:*** *68 w/w-%,* ***C:*** *36 w/w-%, and* ***D:*** *13 w/w-% ε-CL in toluene. The intensity of the highest peak in each spectrum was set to 100%. Lines are shown to distinguish the different distributions.*

In summary, it can be postulated that concentrated systems, e.g. bulk conditions, have a clear effect on the rate of polymerization, most probably due to transport limitation of the monomer by an increase in viscosity upon polymerization. When working in diluted systems, transesterification might affect propagation kinetics in e-ROP, although this cannot be concluded from the rate of monomer consumption. Nevertheless, the effect of transesterification can be visualized with size exclusion chromatography (SEC), as the molecular weight distribution tends to broaden as a result of chain transfer, and with MALDI-ToF-MS, due to the formation of polymers with an other end-group functionality (cyclic polymer formation). In practice, it is possible to minimize intramolecular transesterification in polymerization reactions in general by increasing the monomer concentration.

2.4. *Role of water*

The origin of water

In e-ROP, control over the amount of water is reported to play a crucial role.[26] Although usually control over the polymeric end-groups is required, water present in the system can act as a nucleophile creating carboxylic acid end-functionalized polymer chains, rather than the desired specific functionality that is introduced by an initiator (Figure 2-6). Additionally, an increase of nucleophile concentration also implies the reduction of the molecular weight.

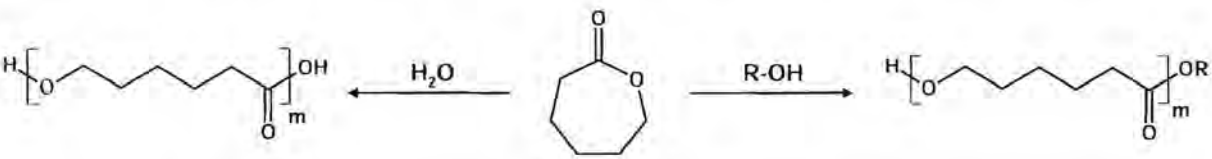

Figure 2-6: *Water can act as nucleophile forming linear carboxylic acid end-functionalized polymer chains.*

Nevertheless, water can not be removed completely from the enzyme, as the enzyme needs some water for maintaining its active conformation.[41-43] From DSC, it was estimated that *Candida antarctica* Lipase B (Novozym 435) needs approximately 250 molecules of water per enzyme molecule to maintain its active conformation.[42] Lee et al. stated that enzymes contain water on three different levels (equation 2.3).[41] The first hydration state is tightly bound, non-exchangeable water (**B1**). This refers to water that stabilizes the enzyme's active conformation by hydrating all polar and ionic groups. The second state represents loosely bound water present in the enzyme (**B2**). It is assumed that this water causes complete coverage of hydrogen-binding sites, and forms a monolayer on all non-polar surfaces. As it binds only weakly to the enzyme, it can be exchanged with the third state, 'free' water (**f**), i.e. water without a specific function. The studies that led to this theory on hydration states were

performed for free enzyme in an organic solvent. Nevertheless, it can be expected that, when applying immobilized enzyme, even more water will be introduced into a system, depending on the hygroscopic character of the carrier material. In conclusion, if e-ROP is used in the synthesis of complex architectures, control over the polymerization will be of crucial importance. Therefore, control over the amount of water is essential and may be obtained by treating the enzyme before use.

$$(H_2O)_{B1} \not\longleftarrow (H_2O)_{B2} \longleftarrow (H_2O)_f \qquad (2.3)$$

Enzyme treatment

Different enzyme treatments have been investigated in order to reduce the amount of water in the enzyme, without losing enzyme activity (Table 2-1 and Figure 2-7). Both chemical ($MgSO_4$, Na_2SO_4 and $CaCl_2$) and physical (reduced pressure, temperature and molecular sieves) drying methods were applied. As water is removed from the enzyme, it is important to know the residual enzyme activity. By performing an enzymatic polymerization of ε-CL, initiated by benzyl alcohol as a model polymerization, information on the residual enzyme activity was obtained. In addition, the amount of water-initiation, i.e. the presence of carboxylic acid end-functionalized PCL species in the total of linear polymer, was quantified by modifying all polymer end-groups with oxalyl chloride and subsequent ^{1}H-NMR-spectroscopy[44] (explained in the Section 2-7). Procedure **1** in Figure 2-7 is taken as a reference, as this contains untreated enzyme. In a typical e-ROP using the untreated enzyme in a reaction performed in an open vessel, the conversion of ε-CL was 95% after 60 mins at 60 °C (black bars). Using Karl Fischer Coulometry (KFC), 0.271 mg water per gram reaction mixture was titrated, which corresponds to 1.12 mg of water in the total reaction mixture. In the final polymer the water-initiation level was just over 52% (white bars). This implies that over half of the linear polymer chains were endcapped with a carboxylic acid end-group.

Table 2-1: *Overview of the enzyme drying procedures, evaluated in Figure 2-7. All procedures were applied for 16 hours.*

Procedure	Drying method
1	at RT (opened)
2	P_2O_5 at RT
3	at 130 °C
4	at 160 °C
5	*in vacuo* at 50 °C
6	*in vacuo* at 100 °C
7	*in vacuo* at 50 °C with act. molecular sieves
8	*in vacuo* at 100 °C with act. molecular sieves
9	P_2O_5 at RT with dried $MgSO_4$
10	P_2O_5 at RT with dried Na_2SO_4
11	P_2O_5 at RT with dried $CaCl_2$

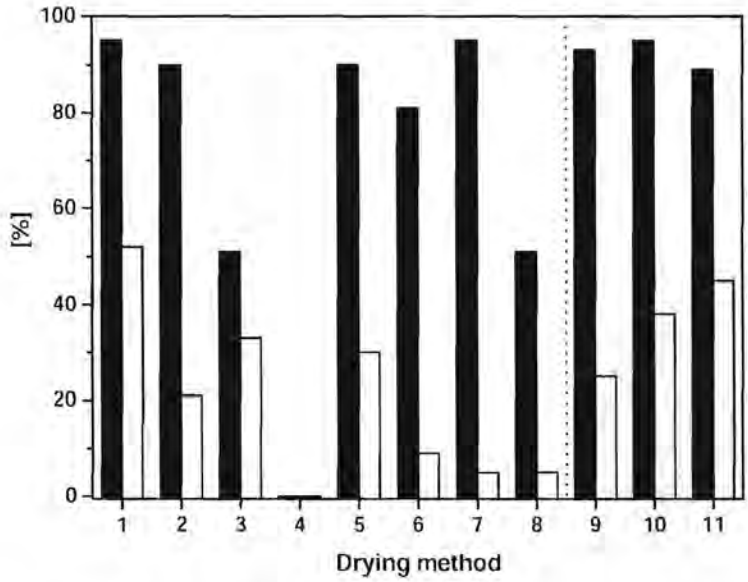

Figure 2-7: *The influence of different enzyme drying methods on the monomer conversion (black) and formation of carboxylic acid end-functionalized PCL (white); monomer conversion was determined with ^{1}H-NMR, and the formation of carboxylic acid end-functionalized PCL was monitored with ^{1}H-NMR after end-group modification with oxalyl chloride; numbers refer to drying procedures listed in the table (all drying procedures were applied for 16 hours).*

As benzyl alcohol was consumed for 90% after 60 mins, it can be calculated how much water in the untreated enzyme participated in the reaction: it corresponds with 1.96 mg water (1.31 w/w-% water in enzyme). This value suggests that the enzyme itself contains a high concentration of water that can initiate polymer chains. Possibly, the reaction medium also attracts water from the air, as the amount of water in the polymer has almost doubled in comparison to the amount of water that was initially present in the system (KFC).

In procedures **2**-**8**, the enzyme was treated according to physical drying methods, such as applying vacuum, high temperature, and the addition of pretreated molecular sieves. As shown in Figure 2-7, the activity decreased dramatically, when (too) high temperatures were applied (procedures **3** and **4**). When drying the enzyme at 160 °C for 16 hours, the enzyme was even completely deactivated. When vacuum was applied for 16 hours, the activity of the enzyme slightly reduced, whereas the amount of water-initiation became considerably lower (procedures **5** and **6**). When the molecular sieves were added to the flask after enzyme treatment at 50 and 100 °C *in vacuo*, but before addition of the stock solution of ε-CL and initiator in toluene, enzyme activity was retained. The amount of water-initiation was drastically reduced to well below 5%, which was set as the detection limit using ^{1}H-NMR-spectroscopy and end-group modification using oxalyl chloride (procedures **7** and **8** respectively). In addition, also chemical treatments were applied in order to dry the enzyme. In procedures **9**-**11**, respectively dried $MgSO_4$, Na_2SO_4 and $CaCl_2$ were used in the reaction mixture to dry the enzyme. The enzyme itself was treated exactly the same as in procedure **2**.

The results clearly show the lack of drying capacity of these 'drying agents' for water present in the enzyme. In conclusion, procedure **7** is the most powerful drying method, taking the combination of activity and water initiation into account.

To check the actual amount of water present in the enzyme before and after drying, thermogravimetric analysis (TGA) was performed. In Figure 2-8A, the TGA-traces of two different batches of Novozym 435 are depicted (**I** and **II**). For both samples, two weight loss transitions are visible, which are both assumed to originate from the release of water. Initially, weight loss is observed in the temperature range until 90 °C, and it is considered to be free water present in the lipase, and water that was adsorbed to the hygroscopic support. In both enzyme batches (**I** and **II**), the weight loss due to the release of this water in untreated enzyme was approx. 0.75 w/w-%. Upon further heating in TGA, the remainder of the water (bound water) was released (0.40 w/w-% for batch **I** and 1.20 w/w-% for batch **II**). Based on this assumption, it was concluded that the amount of bound water differs per batch of enzyme, possibly as the exact amount of immobilized enzyme per bead may differ to a large extent. In addition, the support material may be non-uniform per produced batch. When Novozym 435 is treated according to procedure **7**, TGA-traces show that free water is still removed below 100 °C (Figure 2-8B, **III**). Only 0.11 w/w-% of this water was still present in the treated enzyme. Only a small difference of 1.11 w/w-% was observed in the release of tightly bound water. Therefore, by careful optimization, a drying method was obtained by which most of the free water was removed, while maintaining enzyme activity.

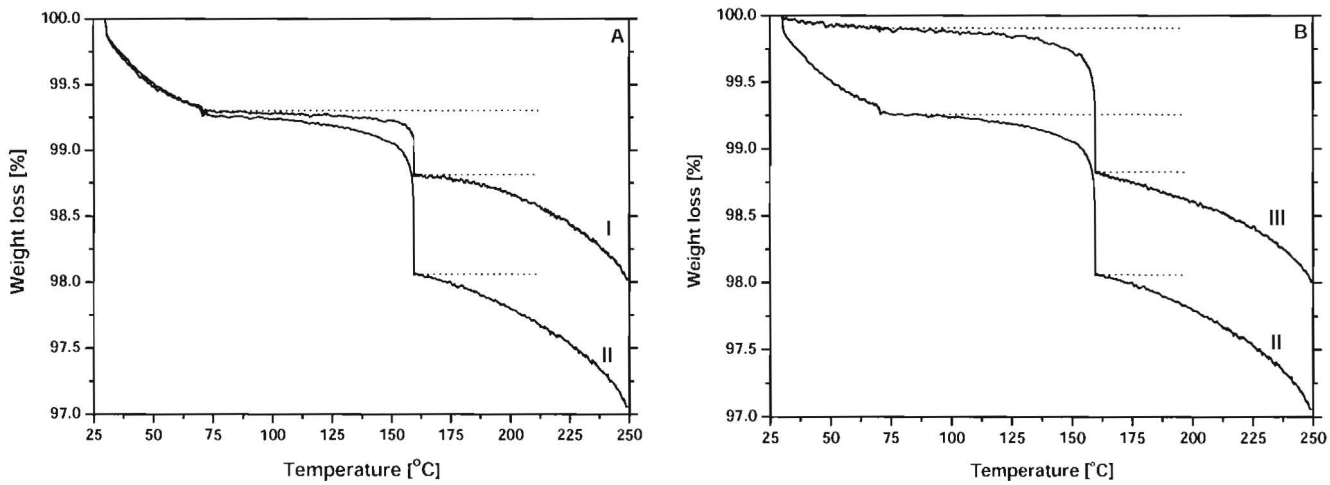

Figure 2-8: *Thermogravimetric Analysis (TGA) of* **A:** *two different batches (**I** and **II**) of untreated enzyme;* **B:** *untreated (**II**) vs. treated enzyme (**III**) from the same batch.*

Table 2-2: *Results of e-ROP of ε-CL from HEBI in toluene with different water concentrations.*

Entry	Titrated water[1] [mg H_2O/g sample]	ε-CL conversion[2] [%]	M_n[3] [g/mol]	M_w/M_n[3] [-]	Water initiation[4] [%]
1	0.318	91	5500	1.95	40
2	0.013	88	6350	2.30	5

Entry **1**: untreated Novozym 435; Entry **2** treated Novozym 435 (procedure 7); [1] determined with Karl Fischer Coulometry from an initial reaction sample; [2] determined with ^{1}H-NMR after 120 mins; [3] determined with SEC on precipitated polymer; [4] determined on precipitated polymer with ^{1}H-NMR after end-group modification with oxalyl chloride.

Enzymatic polymerization under dry conditions

The effect of enzyme treatment on e-ROP of ε-CL initiated by HEBI was investigated by performing polymerizations with untreated and treated Novozym 435 (procedure **7** in Table 2-1) at 60 °C for 120 minutes, respectively entries **1** and **2** in Table 2-2. Directly after the start of the polymerization, a sample was withdrawn and the water present in this sample was determined with Karl Fischer Coulometry (KFC). It was observed that when using untreated Novozym 435 (entry **1**), 0.318 mg/g water was determined in the reaction sample. As all liquid reactants and the solvent were extensively dried, this water must have originated from the enzyme. When the enzyme was treated according to procedure **7** (entry **2** in Table 2-2), only 0.013 mg/g water was determined.

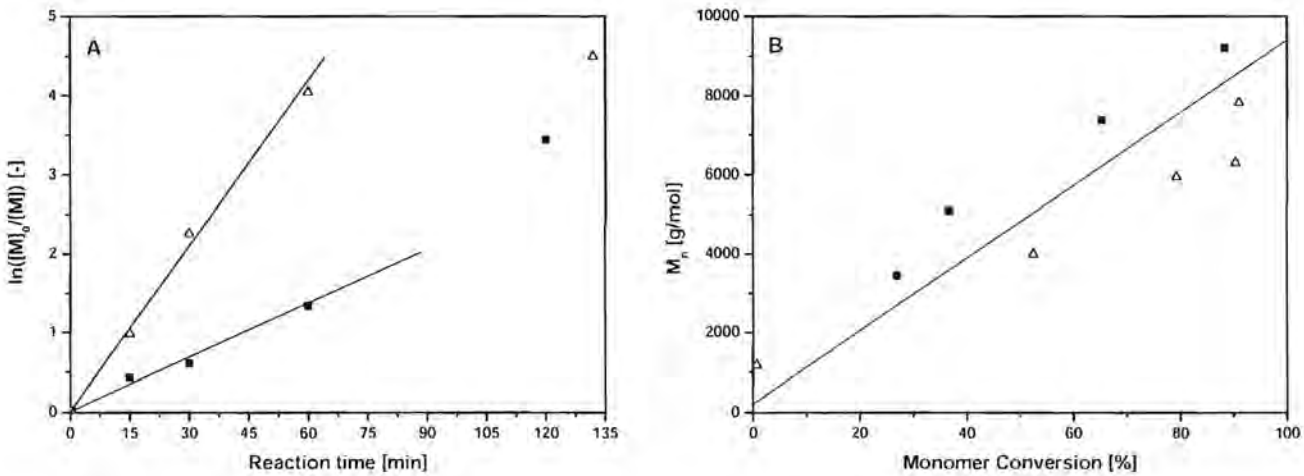

Figure 2-9: A: *Evolution of monomer consumption as a function of reaction time in e-ROP of ε-CL from HEBI with different amounts of water in the enzyme. Monomer consumption was determined with ^{1}H-NMR. Straight lines represent pseudo-first order kinetics;* **B:** *Evolution of molecular weight, M_n, as a function of ε-CL conversion. M_n was determined by ^{1}H-NMR; straight line represents M_n(th.); △: entry **1** (untreated enzyme); ■: entry **2** (treated enzyme).*

The monomer conversion after 120 mins was observed to be almost identical, but the evolution of monomer consumption during the reaction was different (Figure 2-9A). When more water was present, the initial rate of monomer consumption was significantly higher. At the same time, the observed M_n was lower, which can be expected as water adds to the initiator concentration (Figure 2-9B). The polydispersity index was somewhat lower in the presence of water.

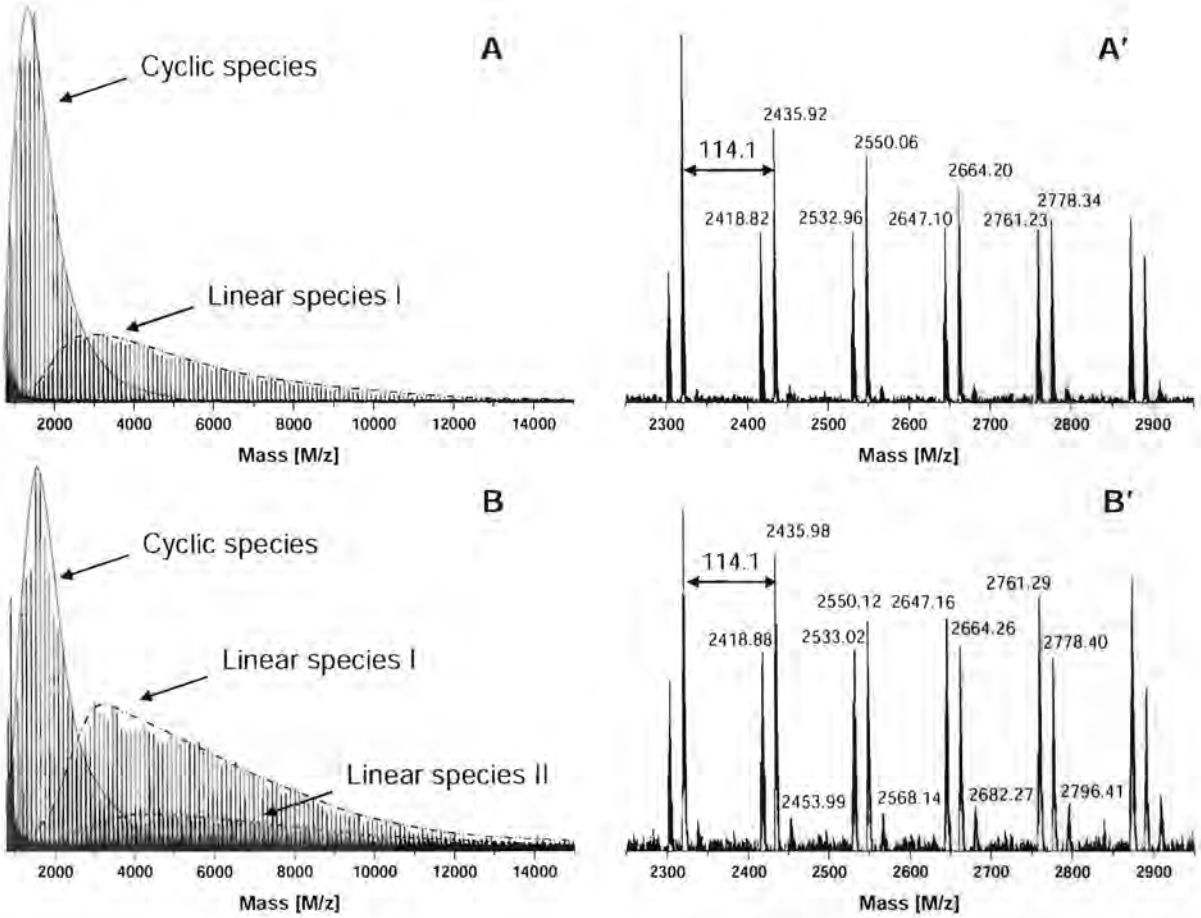

Figure 2-10: *MALDI-ToF-MS traces of PCL obtained from e-ROP of ε-CL from HEBI in toluene with different amounts of water in the enzyme;* **A:** *full spectrum of the polymer obtained from entry* ***2*** *(Table 2-2);* **A':** *the spectrum of the polymer obtained from entry* ***2****, zoomed in on specific peaks (2250-2950 g/mol);* **B:** *full spectrum of the polymer obtained from entry* ***1*** *(Table 2-2);* **B':** *the spectrum of the polymer obtained from entry* ***1****, zoomed in on specific peaks (2250-2950 g/mol); Lines are shown to better visualize the different distributions.*

After precipitation, the water-initiation level was determined. It was observed that the polymer obtained from entry **1** consisted of 40% of chains with carboxylic acid end-groups compared with only 5% when the enzyme was treated according to procedure **7**. Although ^{1}H-NMR spectroscopy revealed the presence of high amounts of water-initiated species in the final polymer (entry **1**), MALDI-ToF-MS did not show these high amounts (Figure 2-10A and B). Nevertheless, upon closer analysis of the spectra (Figure 2-10A' and B'), a third

distribution of peaks, linear species II can be observed in the spectra of both entries **1** and **2** (in addition to cyclic and linear I = HEBI-end-functionalized polymer species). Again, this is an indication that MALDI-ToF-MS cannot be used as a quantitative technique.

In conclusion, it was observed that the rate of monomer consumption was far higher when the enzyme contained more water than when treated enzyme was used (procedure 7). Accordingly, the M_n of the obtained polymer was lower than the theoretical M_n, and more water-initiated polymer species were present in the final polymer yield. The latter is important to keep in mind when aiming for well-defined end-functionalized polymer structures. In addition, the initial activity of the enzyme was observed to be lower when the enzyme was treated (Figure 2-9A). However, on the timescale of the experiment (120 mins), the final conversion of monomer is similar (88% when treated, 91% when untreated).

2.5. Discussion

The proposed mechanism for e-ROP of lactones (Figure 2-2), is based on an 'activated monomer mechanism'. In an activated monomer mechanism, the catalyst (enzyme) only activates monomer species. As enzymes can also activate polymer chains, transesterification reactions, such as chain transfer reactions and cyclic polymer formation, become possible (Figure 2-3). Upon polymerization, monomer is consumed and the concentration of ester bonds in monomer decreases, enabling more polymeric ester bonds to be activated. This process is called product-inhibition, as a lower number of active sites are available for monomer to be activated. Moreover, as polymeric ester bonds are in a transoid conformation (whereas ε-CL ester bonds are in a cisoid conformation), the enzyme activates polymer rather than monomer.[45] Hence, an enzymatic reaction cannot be controlled in terms of the evolution of number average molecular weight and the polydispersity index.

Generally, controlled polymerization techniques do not follow an activated monomer mechanism, but it is the growing polymer chain that is activated. In the case of controlled radical techniques, reversible activation and deactivation by metal halides (ATRP), nitroxide-moieties (NMP), and di- and trithioesters (RAFT) can make the propagating polymer chain grow in a well-defined manner. In ring-opening polymerization of lactones with metal complexes, the polymer chain is activated by the metal and the monomer is incorporated by an insertion mechanism. Only, ROP with an organocatalyst is proposed to obey an activated monomer mechanism, and good control over the reaction kinetics and low polydispersities has been reported.[13] Typically, these two chemical ROP techniques do not seem to activate large cyclic esters (e.g. ω-pentadecalactone) to high conversions or molecular weights. Probably, this selectivity towards small cyclic molecules can be explained by the ring-strain in these monomers. In addition, it could also explain the well-defined polymers that are

formed: In both large cyclic esters and polyesters, the ester bonds have a transoid conformation and reduced ring strain, hence, the chemical catalyst cannot activate these.[46]

The contrast of these controlled polymerization techniques and enzymatic ring-opening polymerization is obviously large, considering the different control over the reaction kinetics, and the molecular weight distribution of the obtained polymer species. Hence, one could argue about the control that can be obtained in e-ROP. Nevertheless, kinetic control is not the only type of control that can be obtained in a reaction. Enzymes are well-known for their enantioselectivity in organic reactions (dynamic kinetic resolution).[47] This principle can also be applied in e-ROP, which can be used in the synthesis of enantiopure polymers.[48] Due to their conformation, enzymes can have a very specific preference for substrates, even if the chiral centre is not adjacent to the ester bond (e.g. 4-methylcaprolactone). In addition, enzymes can be used in regio- and stereoselective polymerizations and modifications. Hence, polymer backbones can be selectively grafted enzymatically in one step, whereas chemical techniques would lack any selectivity and ultimately create crosslinked networks, unless protection and deprotection steps would be implemented.[49]

2.6. Conclusions

This chapter started with the question whether enzymatic ring-opening polymerization can be placed in the scope of available controlled polymerization techniques. In order to answer this question, the proposed mechanism of e-ROP was discussed and it can be concluded that this technique cannot be used as a controlled polymerization technique in terms of reaction kinetics and molecular weight distribution, as the polymer is preferred over monomer in the activation process by the enzyme. Subsequently, the intrinsic side-reactions in e-ROP were investigated. First, the formation of cyclic polymer by intramolecular transesterification: transesterification can cause chain transfer and the formation of cyclic polymer. The former has a dramatic influence on the molecular weight distribution, whilst the latter affects the end-group functionality of the polymer. Using MALDI-ToF-MS, it was demonstrated that bulk conditions should be chosen in order to minimize the formation of cyclic polymer. Second, water has a dual role in e-ROP: enzymes need water to retain their active conformation, but at the same time water can act as competitive nucleophile towards a (functional) initiator, thereby forming polymer chains that lack the specifically introduced end-group functionality of the initiator. The major source of water in the system was found to be the enzyme. The rest of the water can be removed by applying physical drying techniques, without losing enzyme activity.

In conclusion, e-ROP can be applied as a synthetic tool in polymer chemistry. Although kinetic control is not obtained, enzymes do provide specific advantages over conventional

catalysts and polymerization techniques: enzymes are biocatalysts that can catalyze polymerization without the need for a toxic metal-catalyst. Moreover, the polymers that are synthesized enzymatically, are expected to degrade. In addition, they are praised for their natural enantioselectivity, and claimed to be regio- and stereoselective catalysts.[49] Finally, enzymatic reactions can be performed under very mild conditions, which can be an advantage in the synthesis of chemically unstable polymeric materials.[50]

2.7. Experimental section

Materials

All chemicals were purchased from Aldrich, stored over molecular sieves and used without further purification unless otherwise noted. Toluene (purchased from Biosolve) was dried over alumina and stored over molecular sieves (3Å). ε-Caprolactone (ε-CL) was distilled over CaH_2 and stored over molecular sieves. Novozym 435 was obtained from Novozymes A/S, Denmark. Molecular sieves (3Å) were dried in an oven at 420 °C prior to use.

Methods

The water concentrations of the reaction media were determined by Karl-Fischer Coulometry using a Mettler Toledo DL32 Coulometer without diaphragm and Apura CombiCoulomat fritless (Merck) as electrolyte. Size exclusion chromatography (SEC) was performed on a Waters Model 510 pump and Waters 712 WISP, using PL-gel mix D columns (300 x 7.5 mm, Polymer Laboratories) at 40 °C, and detection was performed using a Waters Model 410 refractometer and a Waters 486 UV-spectrometer. THF was used as eluent with a flow rate of 1.0 mL/min. All samples were diluted to 1.0 mg/mL in THF and filtered using 0.2 μm syringe filters. The molecular weights of all polymers were calculated based on universal standards, using the Mark-Houwink-Sakurada relationship (K = 0.00109 dL/g and a = 0.600).[51] ^{1}H-NMR spectroscopy was performed using a VARIAN 400 NMR at 20 °C. Samples were diluted in $CDCl_3$ to 30-50 mg/ml. Data were acquired using VNMR-software. MALDI-ToF-MS analysis was carried out on a Voyager DE-STR from Applied Biosystems using trans-2-[3-(4-tert-butylphenyl)-2-methyl-2-propenylidene]-malononitrile (DCTB) as matrix material and potassium trifluoroacetate as ionization agent. All spectra were recorded in the reflector mode. Samples were prepared using 1 mg/mL of polymer in THF. The ratio of polymer sample to matrix was 1 : 5 (w/w/%). Thermogravimetric analysis (TGA) was performed on a Perkin Elmer Pyris 7 TGA with N_2 as purge gas. The applied temperature profile was (equation 2.4). Data was processed with TA-Universal Analysis.

$$30 \xrightarrow[1\,min]{5°C/min} 70 \xrightarrow[5\,min]{5°C/min} 160 \xrightarrow[5\,min]{25°C/min} 300 \xrightarrow{1\,min} \quad (2.4)$$

Synthesis of 2'-hydroxyethyl-2-bromoisobutyrate (HEBI)

Triethylamine (TEA; 3.871 g, 38.3 mmol) and ethylene glycol (EG; 2.484 g, 40.0 mmol) were dissolved in 150 mL THF in a 500 mL round bottom flask, which was placed in an ice bath. A solution of 2-bromoisobutyryl bromide (2-BIBB; 8.637 g, 37.5 mmol) and THF (50 mL) was added to a dropping funnel and slowly added to the flask. The formed HBr was neutralized by TEA, which precipitated as a salt. After all 2-BIBB had been added to the flask, the heterogeneous mixture was kept stirring for one more hour to let all 2-BIBB react. The salts were then filtrated and the THF was removed by evaporation *in vacuo*. To the resulting yellowish oil HCl (1M, 125 mL) was added and then extracted with dichloromethane (3 x 50 mL). Subsequently, the organic phase was washed with water, saturated Na_2CO_3-solution, and saturated NaCl-solution (3 x 200 mL), and dried with $MgSO_4$. After filtration, dichloromethane was evaporated *in vacuo* to give a mixture of the di-substituted and mono-substituted compound. **An analytically pure sample was obtained by column chromatography (SiO_2; 3/1 dichloromethane/heptane as the eluent). ^{1}H-NMR ($CDCl_3$, δ): 1.94 (s, 6H, -C(CH_3)$_2$), 2.08-2.20 (s, 1H, -O*H*), 3.86 (m, 2H, -CH_2OH), 4.30 (m, 2H, -CH_2OC=O).**

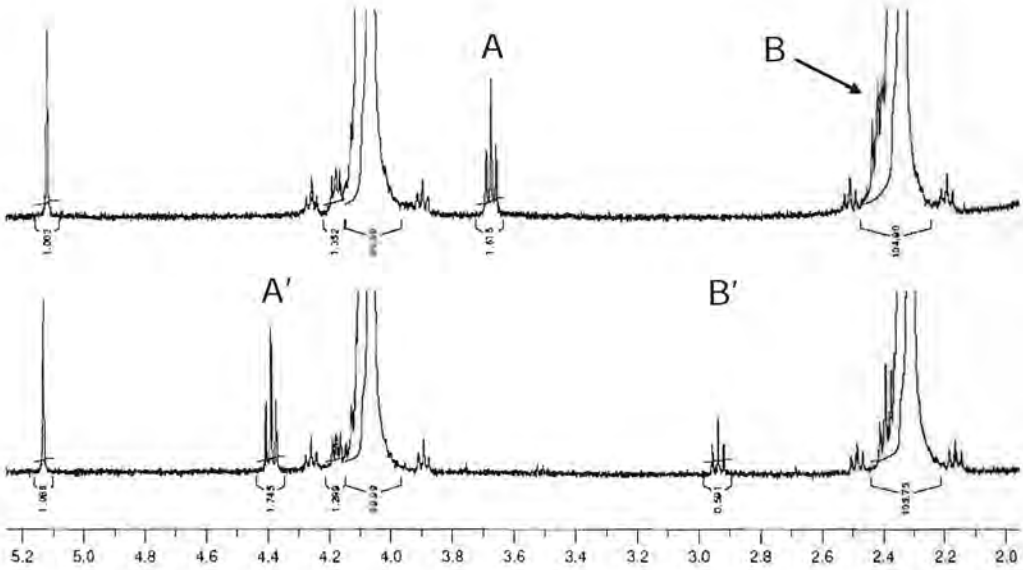

Figure 2-11: *^{1}H-NMR-spectra of PCL-BA (precipitated polymer) before and after reaction with oxalyl chloride.*

Determination of the extent of water-initiation

The amount of water-initiated polymer chains in the polymer was determined by ^{1}H-NMR, by comparing the methylene-protons (-C*H*$_2$-OH) of one end-group with the methylene-protons (-C*H*$_2$-COOH) of the other end (as depicted in Figure 2-11).[44] As in the original polymer spectrum, the latter protons coincide with the repeating methylene-ester protons of the polymer. Modification of all carboxylic acid and hydroxyl end-groups was performed with

oxalyl chloride, which converts the hydroxyl groups, shifting the neighboring methylene peak from δ = 3.65 to δ = 4.40 ppm (A→A'), and the carboxylic acid groups into acid chloride moieties, shifting the neighboring methylene peak from δ = 2.35 to δ = 2.90 ppm (B→B'). In order to convert all end-groups, a few drops of oxalyl chloride were added to a NMR-tube containing the polymer in $CDCl_3$ and allowed to react for 30 mins.

Enzyme pretreatment

An exact amount of Novozym 435 (10 w/w-% to monomer) was weighed into a flask together with a magnetic stirrer bar. The enzyme was then dried according to the following procedures (numbers relate to Table 2-1 and Figure 2-7):

1 Reference sample; the enzyme was used as received, and the reaction was performed in an open system with dried reactants;

2 A flask with enzyme was placed in a desiccator with P_2O_5 for 16 hours;

3/4 A flask with enzyme was placed in an oven at respectively 130 and 160 °C for 16 hours;

5/6 A flask with enzyme was placed *in vacuo* at respectively 50 and 100 °C for 16 hours;

7/8 A flask with enzyme was placed *in vacuo* at respectively 50 and 100 °C for 16 hours, and activated molecular sieves (3Å) were added to the flask after enzyme treatment, but prior to the reaction;

9/11 Procedure 2 was followed, and, respectively dried $MgSO_4$/Na_2SO_4/$CaCl_2$ was added to the flask after enzyme treatment.

Enzyme activity test

For all model polymerizations, the amount of Novozym 435 with respect to monomer was kept constant at 10 w/w-%. A stock solution of 2'-hydroxyethyl-2-bromoisobutyrate (HEBI) (160 mg; 0.034 M) and ε-CL (13.921 g; 2.812 M) in toluene (24.951 g) was prepared and stored over molecular sieves. The reaction was started by adding 3.900 g of the stock solution to a flask containing (un-)treated enzyme (and molecular sieves) and followed by heating in an oil bath to 60 °C. Immediately after the start of the reaction, a sample (~0.4 mL) was withdrawn from the reaction mixture and analyzed using Karl-Fischer Coulometry. At specified time intervals further samples (~0.15 mL) were withdrawn from the reaction mixture with a syringe and analyzed by ^{1}H-NMR spectroscopy and SEC to determine monomer conversion and the molecular weight distribution, respectively. After 60 minutes, the reaction mixture was dissolved in dichloromethane and the enzyme removed by filtration. The polymer was precipitated by adding it dropwise to cold methanol. The precipitate was dried overnight *in vacuo* at 50 °C, resulting in a final yield of 0.89 g (70%). The precipitated polymer was analyzed by ^{1}H-NMR spectroscopy, SEC, and MALDI-ToF-MS to obtain more insight into the polymer product.

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3

Quantification of Polymer Species with Different End-groups using Critical Chromatography

Abstract

The enzymatic ring-opening polymerization (e-ROP) of ε-caprolactone (ε-CL) to poly(ε-caprolactone) (PCL) employing Novozym 435 as the catalyst was studied in detail. Depending on the applied reaction conditions, cyclic and linear polymer structures are created in different amounts, and, if an additional initiator is used, linear polymer chains with different end-groups are formed. An easy method to prove the presence of the different species is MALDI-ToF-MS, which has enough resolution to distinguish individual polymer molecules. Nevertheless, this technique can only be used qualitatively due to mass discrimination effects. In the present study, liquid chromatography under critical conditions (LCCC) is applied to separate and quantify PCL with different end-groups. It was observed that if untreated, i.e. non-dried, enzyme is used, up to 40 w/w-% of hydroxy-acid species can be formed. When aiming for end-functionalized polymer, the size and type of the nucleophile that is used as initiator, determine the initiation efficiency. Monomer concentration was found to be the primary tool in reducing the formation of cyclic polymer species. Polymerization under bulk conditions suppressed intramolecular transesterification reactions (<20 w/w-% cyclic species), whereas in highly diluted systems (0.7 M), up to 70 w/w-% of cyclic species were observed.

3.1. Introduction

In the previous chapter, a detailed study of enzymatic ring-opening polymerization (e-ROP) of ε-CL was discussed. In addition, important parameters in e-ROP, such as the amount of water and cycle formation, were investigated and optimized for the e-ROP of ε-CL. Yet, it is still not completely understood what exactly occurred on the molecular level at different stages during the polymerization. Polymer species bearing carboxylic acid end-groups were found in the final polymer, but were these species formed in the initial stages of the reaction, as water was close to the enzyme, or where they only formed over time, due to release of water from the enzyme carrier material? It is known that cyclic polymer species are entropically favorable over linear polymer species, and hence will be formed over time (Figure 3-1).[1, 2] But are cyclic polymer species also formed due to the mechanism, or will they be formed at the initial stages of the reaction?

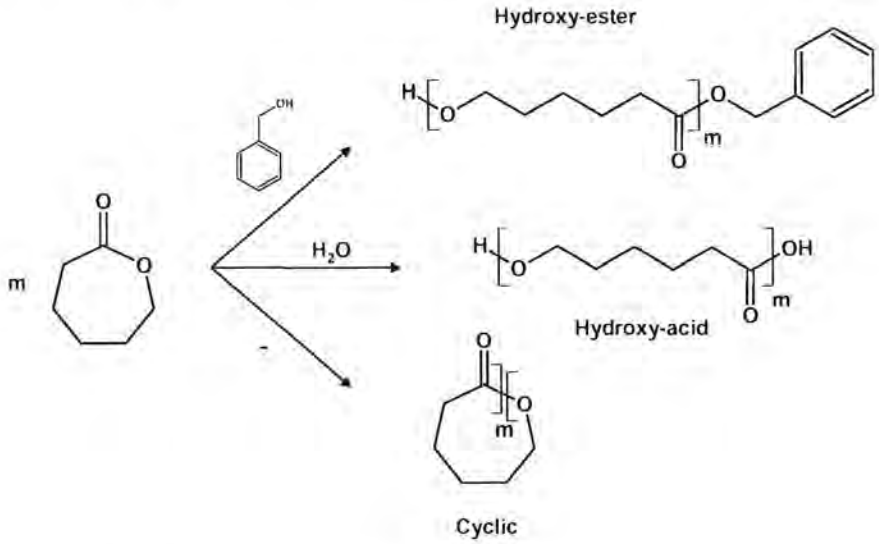

Figure 3-1: *Typical PCL species obtained in e-ROP of ε-CL: cyclic polymer species, linear water-initiated polymer species (hydroxy-acid), and linear 'initiator'-initiated polymer species (hydroxy-ester).*

End-group analysis on polymers in reaction samples may provide an answer to these questions as detailed information becomes available on the presence of different polymer species. Typically, end-group analysis on polymers can be achieved by spectrometric techniques, such as electrospray ionization mass spectrometry (ESI-MS) or matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-ToF-MS).[3, 4] The latter has been applied to study enzyme-catalyzed transesterification reactions.[5] A major drawback of spectrometric techniques is that they can only be used in a qualitative manner, since several factors affect the intensity of the MS-signal, e.g. specific sample properties such as molecular weight, molecular weight distribution, end-groups and the nature of the polymer. Nevertheless, spectrometric techniques have been applied for quantitative use in polymer

characterization, even in this study. It has been claimed that this would be applicable, if a set of specific polymer standards is available, an internal standard is used, and only after careful optimization.[6-9] Moreover, techniques such as end-group titration[10, 11] and end-group modification in combination with ^{1}H or ^{31}P-NMR spectroscopy can be applied to obtain insight into the end-group functionality.[12, 13] While these techniques do provide quantitative data, they only take linear polymer species into account and data becomes less accurate when applying higher molecular weight polymer. Additionally, they cannot provide any insight into how different end-groups are combined in one and the same polymer molecule.

Liquid chromatography under critical conditions (LCCC) has been applied for the characterization of specific functionalities in polymers.[14] Typically, it is used to obtain insight into the end-group distribution or functionality of the polymer, but with increasing molecular weight, the parameters will become more and more inaccurate.[15, 16] As the molecular weight becomes too high, LCCC cannot reliably be used to provide quantitative information on the polymer end-group.[17] LCCC on oligo-caprolactonediols has been reported in literature, but no cyclic structures were considered/present[18], and the analysis was performed under normal phase conditions.[19] We have chosen to investigate and elaborate on the technique of LCCC in this chapter to obtain more mechanistic insight into the actual process of enzymatic ring-opening polymerization. As specific standards with different molecular weights are not available for the specific PCL, a method had to be developed

3.2. Theoretical background

Critical conditions

Liquid chromatography (LC) is widely utilized for (polymer) characterization, due to its broad variety of solvating capabilities with great scope for selectivity optimization[20] Several parameters can be chosen and changed in order to optimize separation based on size exclusion, interaction, and ion-exchange. In polymer characterization, LC is normally applied to obtain insight into the molecular characteristics of the polymer backbone. By bringing the polymer into solution, information such as molecular weight distribution and chemical composition distribution can be obtained due to the mutual interactions between stationary phase (column) and the polymer in the mobile phase (eluent). In Figure 3-2, a schematic representation is depicted of the kind of separation that is obtained, when changing the eluent from a good solvent (size exclusion) to a solvent/non-solvent system (interaction). In the ultimate transition from the size exclusion mode to the interaction mode, the critical conditions are found.

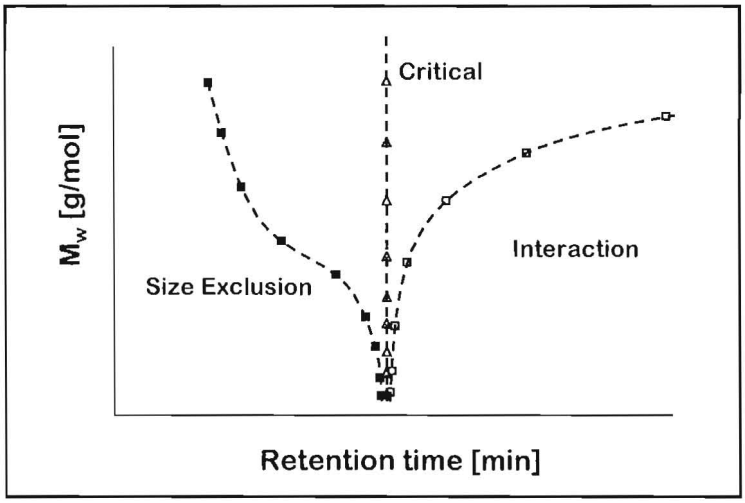

Figure 3-2: *Schematic representation of different modes for separation in liquid chromatography.*[21]

These critical conditions are characterized by the lack of contribution of the backbone-unit to the Gibbs free energy of the polymer, $\Delta G_{polymer}$, and they are only achieved if the enthalpic contribution (due to interaction) of the polymer backbone-unit, $\Delta H_{backbone\text{-}unit}$, equals the entropic term (due to size exclusion) of the polymer backbone-unit, $T\Delta S_{backbone\text{-}unit}$ (equations 3.1 and 3.2).[22] Hence, separation only occurs on the basis of different contributions of the end-groups to the total Gibbs free energy of the polymer, whereas differences in molecular weight and the chemical composition of the backbone do not affect the retention of the polymer at these critical conditions.

$$\Delta G_{polymer} = \Delta G_{endgroup\ 1} + \Delta G_{endgroup\ 2} + n\ \Delta G_{backbone\text{-}unit} \qquad (3.1)$$

$$\Delta G_{backbone\text{-}unit} = \Delta H_{backbone\text{-}unit} - T\ \Delta S_{backbone\text{-}unit} \qquad (3.2)$$

The critical conditions depend on many factors in the analysis, such as eluent composition, temperature, the type of stationary phase and its pore size, and the addition of acid or base[22-24]. These conditions can be obtained for a polymer via different approaches: the most applied route is to choose the stationary phase, flow and temperature, such that by changing the eluent composition (solvent/non-solvent) critical conditions are obtained. Unfortunately, adjustment of these parameters to obtain critical conditions is a delicate process, in particular since some parameters are mutually correlated.

Quantification using Evaporative Light-Scattering Detection

An ELSD-detector consists of a heated tube, in which eluent leaving the column is nebulized. As the solvent is evaporated, dry polymer is directed to a light scattering detector. The concentration of particles is determined by the amount of scattered light. To enable quantification of the separated polymer species with an ELSD-detector, a set of well-defined

polymers with one specific end-group is used as standards to perform a calibration.[12] This calibration is needed as the ELSD-detector exhibits non-linear behavior towards concentration, molecular weight and possibly even to structural differences. These influences must be individually determined before quantification of samples can be achieved.

In general, the relationship between the amount of injected polymer and the obtained area can be expressed as (equation 3.3):

$$\text{Area} = A_0 * m_{inj}^{A_1} \quad , \tag{3.3}$$

in which the area is correlated to the injected mass, m_{inj}, with the constants A_0 and A_1. This can be rewritten and plotted in a log-log diagram of Log(Area) as a function of Log(Concentration), in which $Log(A_0)$ represents the intercept and A_1 the slope:

$$\text{Log(Area)} = \text{Log}(A_0) + A_1 * \text{Log(Concentration)} \tag{3.4}$$

For a set of well-defined polymer standards with one specific end-group, $Log(A_0)$ and A_1 can be easily determined and used for quantification of a dataset of samples of unknown composition. However, it is generally difficult to obtain these well-defined standards for any specific polymer species. Hence, a broader set of ill-defined polymers must be used. In this case, the detector sensitivity of each polymer species must be determined individually in order to allow for structural or chemical differences between the polymer species.

3.3. *Enzymatic synthesis of PCL*

When PCL is synthesized via the initiator method in e-ROP[25], three different polymer species are obtained (Figure 3-1). In order to obtain a better insight into the actual enzymatic process, several enzymatic polymerizations were performed under different reaction conditions. Subsequently, the obtained polymers were separated based on their end-groups and quantified using LCCC. Since the specific PCL-species were not all individually available, a set of ill-defined PCL standards (**X-Z**) was synthesized to determine the critical conditions and the detector sensitivity. All three standards were synthesized via e-ROP, and they possessed different end-group functionalities. **X** was synthesized using 1,6-hexanediol (HD) as initiator, **Y** using water, and **Z** using benzyl alcohol (BA). Subsequently, the reaction conditions of the different enzymatic polymerizations were carefully chosen to obtain more information on the actual process of e-ROP with respect to different initiators, concentrations, and amounts of water (Table 3-1, entries **1-6**).

In Table 3-1, entry **1** functions as a reference sample for the benzyl alcohol-initiated system where the enzyme was treated according to procedure **7** described in Chapter 2. This system is used for comparison with entries **2** and **3**, where the concentrations were altered to bulk and diluted conditions (6x more diluted than **1**), respectively. Entry **4** was similar to the reference, but as the enzyme was not treated, considerably more water was expected to be present. In entry **5** and **6**, bifunctional initiators **I** and **II** were applied (Figure 3-3). Immediately after the start of the reaction, a first sample was taken from which the initial water content in the reaction mixture could be determined using Karl Fischer Coulometry. Typically, samples were taken after predetermined time intervals to monitor the reaction (monomer conversion, molecular weight distribution, water-initiation, and the formation of the three polymer species). After 120 mins the reaction was stopped.

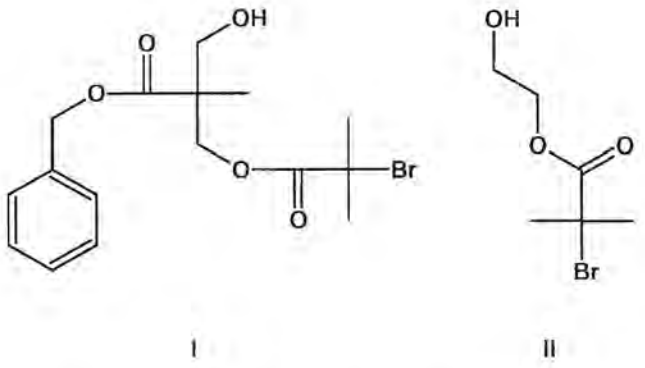

Figure 3-3: *Bifunctional initiators I and II, applied in entry 5 and 6.*

In Table 3-1, the results of the polymerization are shown for the final polymers. It was observed that the initial amount of water in the systems (entries **1-6**) was low, except when untreated enzyme was used (entry **4**). In this case, it would be expected that the amount of water-initiated polymer species, determined by ^{1}H-NMR spectroscopy after end-group modification with oxalyl chloride[26], was the highest (36%). However, this was not the case, as the results show that all final polymers contain large amounts of water-initiated polymer chains (>20%). Typically, the monomer conversion is 50-75% after 120 mins of polymerization. Only in the reaction performed in bulk, sampling was stopped after 60 mins, due to increased viscosity. The number average molecular weight, M_n, of the polymer obtained in entry **1**, was approx. 3500 g/mol, which is lower than expected considering the monomer conversion (76% conversion would correspond to M_n(th.) of 7750 g/mol). This might be an indication that linear hydroxy-acid and cyclic polymer species were present. It was also expected that the molecular weight of the polymer obtained in entry **4**, would be lower than that of the reference, as more water (initiator) was present. However, the molecular weight of the polymer obtained in entry **4** was unexpectedly high.

Table 3-1: *Reaction conditions of e-ROP of ε-CL at 60 °C, and overview of the results of the obtained polymers.*

Entry	Initiator [1]	$[Monomer]_0$ [mol/L]	$[Water]_0$ [mg/g] [2]	Monomer conversion [%] [3]	Initiator conversion [%] [3]	M_n [g/mol] [4]	M_w/M_n [-] [4]	Water initiation [%] [5]
X	HD	4.6	0.035	79	100	4700	2.18	49
Y	water	4.6	0.415	75	100	5600	2.98	100
Z	BA	4.6	0.116	51	97	3700	2.72	16
1	BA	4.1	0.015	76	100	3500	2.58	33
2	BA	(bulk)	0.085	51[a]	100	4650	2.48	34
3	BA	0.7 (diluted)	0.004	59	100	1300	2.31	48
4[b]	BA	4.1 (untreated)	0.235	98	96	4600	2.31	36
5	Ini I	4.1	0.037	70	50	3200	3.30	59
6	Ini II	4.1	0.027	68	94	4350	2.88	20

[1] HD: 1,6-hexanediol; BA: benzyl alcohol; [2] determined with Karl Fischer Coulometry from an initial reaction sample; [3] after 120 mins reaction, determined with ^{1}H-NMR spectroscopy; [4] determined with SEC on the final polymer (universal calibration); [5] ratio of hydroxy-acid : hydroxy-ester in the final polymer, determined with ^{1}H-NMR spectroscopy after end-group modification with oxalyl chloride; [a] sampling was stopped after 60 mins due to high viscosity; [b] untreated enzyme was used.

The most logical explanation for this discrepancy would be that only a fraction of the water present in the system (entry **4**) has initiated polymer chains, and that the treated systems (entries **1-3** and **5-6**) were not as dry as expected. The molecular weight distributions of all polymers are rather broad. The lack of control over the molecular weight distribution that is often observed in e-ROP, might have had several causes, including the batch of enzyme that was used.

3.4. *End-group separation using LCCC*

3.4.1. Establishing critical conditions in LCCC

Our objective was to establish critical LC conditions to separate PCL species with different end-group functionalities: cyclic polymer species (lacking any end-group), linear hydroxy-acid species, and linear hydroxy-ester species (Figure 3.1). Four 250*4 mm Nucleosil 120-5 (Machery-Nagel) C18-columns were placed in series to enhance separation when the critical conditions for PCL were obtained. THF, regarded as a very good solvent for PCL and convenient for LC-use, was chosen as solvent. Water, a non-solvent for PCL, was added to the eluent mixture and, by carefully adjusting the eluent composition, the critical LC-conditions were established. The main concern in finding critical conditions in a solvent/non-

solvent system is that the eluent composition is very close to the cloud point of the polymer. In our case, the critical conditions were obtained in an eluent composition of 82.5/17.5 w/w-% THF/water (Figure 3-4). The water phase consisted of 1.5 vol-% formic acid in order to promote the dissolution of low molecular weight hydroxy-acids species (water-initiated polymer).

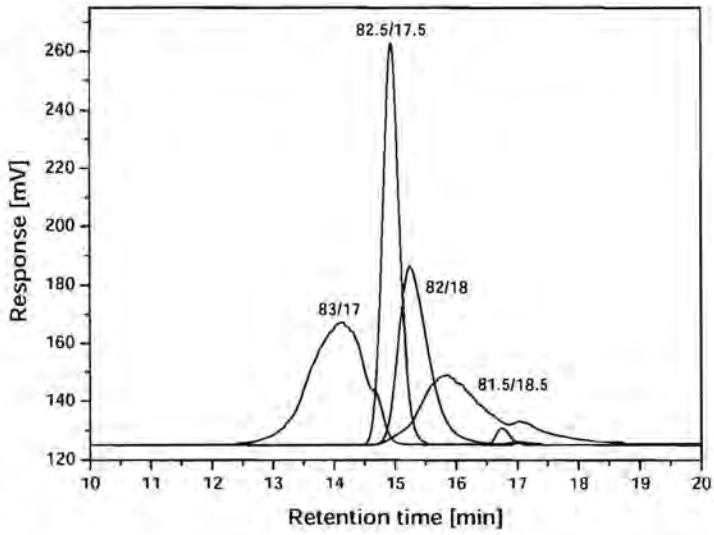

Figure 3-4: *LCCC-chromatograms of PCL (entry **X**), performed with different eluent compositions at 40 °C; ratios in chromatograms represent the eluent composition, THF/water.*

Figure 3-5 shows the LCCC-chromatograms of the final polymers obtained in entries **1-6**. It can be observed that linear hydroxy-acid species eluted first (~14.5-15.5 mins), followed by linear hydroxy-ester species (~15.5-16.5 mins). Finally, cyclic polymer species are eluted (~16.5-17.5 mins). With the exception of entry **5**, good separation of the three polymer species was obtained. This may be due to the fact that a more hydrophobic initiator (initiator **I**) was used in entry **5**, resulting in an overlap of the hydroxy-ester species and cyclic polymer species.

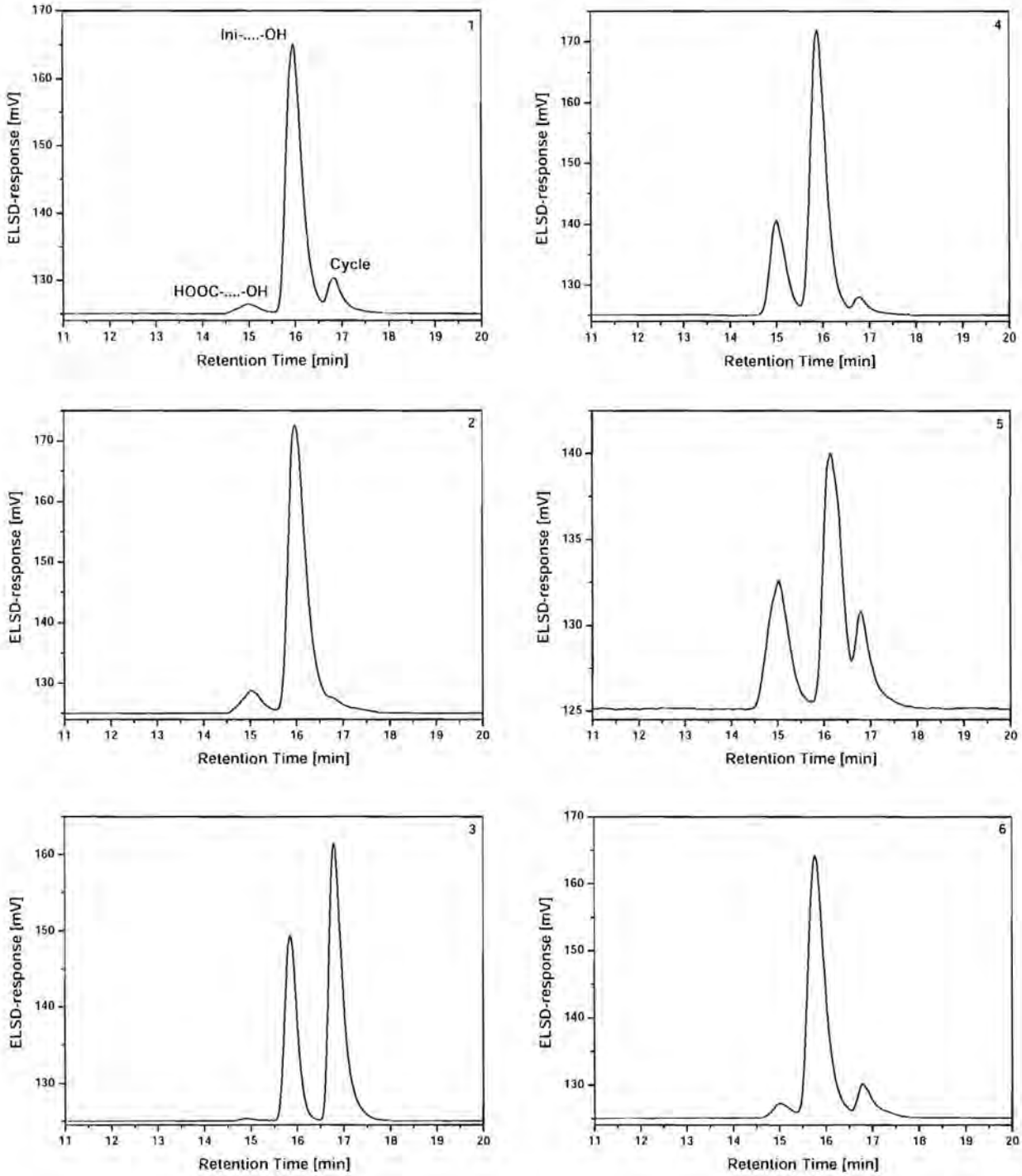

Figure 3-5: *LCCC-chromatograms of the final polymers of entries **1-6**; left peak: hydroxy-acid species; centre peak: hydroxy-ester species; right peak: cyclic polymer species.*

3.4.2. Quantification using evaporative light scattering detector

The availability of detectors when performing LCCC on PCL-polymer is limited. As the polymer has a very weak UV-signal that interferes with the UV-absorbance of THF and formic acid (at 230 nm), a UV-detector is unsuitable. Normally, a differential refractive index-detector (DRI) would be the first choice in LC, but the DRI is very sensitive towards changes in eluent composition. Hence, when working with binary (or ternary) eluent systems,

the DRI-detector cannot be used. The evaporative light-scattering detector (ELSD) is known as a powerful concentration detector in liquid chromatography, if the investigated material is (relatively) non-volatile.[27] Additionally, ELSD-detection is especially applicable when working with binary eluent compositions, since the eluent is evaporated and has no influence on the detection in contrast to other detection techniques. Nevertheless, ELSD is known to be sensitive to concentration and molecular weight, which makes the technique complicated for the quantification of polymer samples of unknown composition and unknown molecular weight distributions per specific component. Moreover, the response of the ELSD might be influenced by the structure of the component. Hence, another detector signal may be expected for cyclic polymer species compared with linear species.

As mentioned previously, it is often difficult to obtain the pure, individual polymer species with similar molecular weight as the reaction samples, especially in the case of cyclic polymer species. In order to enable quantification, polymers were synthesized enzymatically, obtaining mixtures of the desired polymer species with specific end-groups. The exact composition was tuned by the applied reaction conditions. Since the polymer obtained from entry **X** was initiated by 1,6-hexanediol (HD), three components were expected to be formed (linear hydroxy-acid, linear bishydroxyl and cyclic polymer species). In LCCC, no different retention was achieved when trying to separate the two linear species of entry **X** (Figure 3-4), probably due to the apparent small difference in polarity between linear hydroxy-acid and linear bishydroxyl species. Hence, the 'calibration standards' from entries **X** and **Y** consisted of two components (hydroxy-acid and cyclic polymer species), whereas the polymer obtained from entry **Z** consisted of three components (linear hydroxy-acid, linear hydroxy-ester and cyclic polymer species).

It was mentioned before that the ELSD-signal is influenced by concentration, molecular weight, and by structural differences. The effect of the molecular weight is in our systems assumed to be negligible, as all polymers are in the same order of molecular weight (M_n ~ 1000-5000 g/mol). Therefore, an approach was chosen to investigate whether an effect of the structural differences in the polymer compounds, i.e. linear species and cyclic species, on the ELSD-signal could be determined. First, polymers obtained from entry **Z** were injected in different concentrations, obtaining corresponding ELSD-signals (area) for the individual polymer species. When considering the ratio of the concentration of these injections, i.e. the highest concentration is set to 1, a relationship is achieved for the detector signal irrespective of the injected concentration. Figure 3-6 shows the evolution of Log(area) as a function of $-\text{Log}(C/C_{max})$ (as ratio $[C/C_{max}] \leq 0$). It was observed that the linear polymer species have a parallel trend, whereas cyclic polymer shows a different trend. This result suggests that the two linear species have the same detector response, whereas cyclic polymer species show a different response in the ELSD-detector. In practice, this can be explained when the structure of a cyclic polymer is compared with the structure of a linear polymer. It may be expected

that light scattering on particles of cyclic polymer may be different from light scattering on particles of linear polymer chains. In this study, different sensitivity of the ELSD towards linear and cyclic polymer species is assumed and quantification is based on this difference. However, this assumption must be considered with care. Equation 3.4 suggests a linear relationship between Log(Area) and Log(Concentration) whereas this is not observed in Figure 3-6. Moreover, the discrepancy in the Log(Area) of linear hydroxy-acid and cyclic polymer species depicted in Figure 3-6 may not be significant.

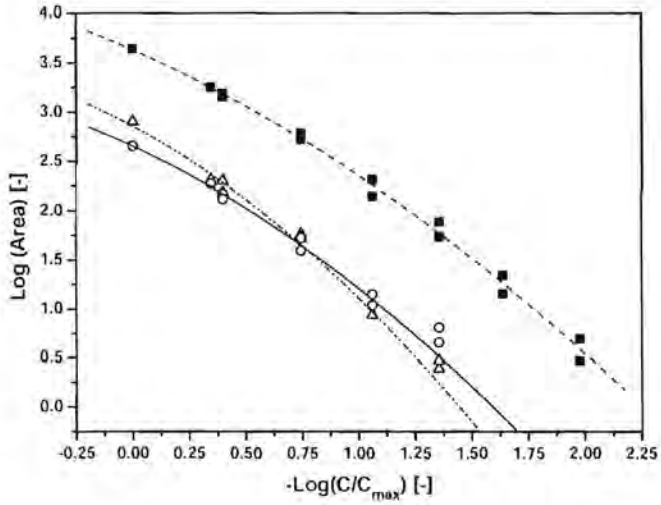

Figure 3-6: *Visualization of the effect of structural differences on the ELSD-signal (area) depicted in a log-log diagram of entry* **Z**. *(C/C_{max}) refers to the ratio of any concentration and the highest concentration;* ■*: linear hydroxy-ester species;* ○*: linear hydroxy-acid species;* △*: cyclic polymer species. Lines reflect the best fit (2nd order).*

The next step in the quantification-process was to determine the detector constants A_1 and $Log(A_0)$ for linear polymer species, and separately for cyclic polymer species. In order to have a larger set of 'standards', and thus a more accurate fit, the final polymer products of the specific enzymatic polymerizations under different conditions (entries **1-6**) were also used for quantification. In order to calculate the detector constants from the data set of polymers, an iterative method was applied, based on the least square method (equation 3.5). In this method, different sensitivity was considered for the linear and cyclic polymer species.

$$\sum_{i=1}^{\infty}\chi_i^2=\sum_{i=1}^{\infty}\frac{\left(\sum_{j=1}^{2}c_{i,j}-c_i^o\right)^2}{c_i^o} \qquad (3.5)$$

Where c_i^o represents the total injected concentration of polymer for standard *i*, and $\Sigma c_{i,j}$ is the sum of the concentrations calculated from $\log(A_0)$ and A_1 and the area below the separated peaks in the chromatogram, and *j* represents each specific polymer species.

$$c_{i,j}=10^{\frac{\log(Area)_{i,j}-\log(A_{0,j})}{A_{1,j}}} \qquad (3.6)$$

Using a spread-sheet program, the minimal $\Sigma\chi_j^2$ and the corresponding $Log(A_{0,j})$ and $A_{1,j}$ for the polymer species, j (j = 1: linear polymer species; j = 2: cyclic polymer species) are easily determined. In Table 3-2, the values of the detector constants are listed for linear and cyclic polymer species.

Table 3-2: *Values of the detector constants $Log(A_0)$ and A_1 for linear and cyclic polymer species, assuming equal sensitivity for the linear components.*

j	Polymer species	Detector constants	
		$Log(A_0)$ [-]	A_1 [kg/mg]
1	Linear	-1.33987	1.19429
2	cyclic	-6.58892	2.48802

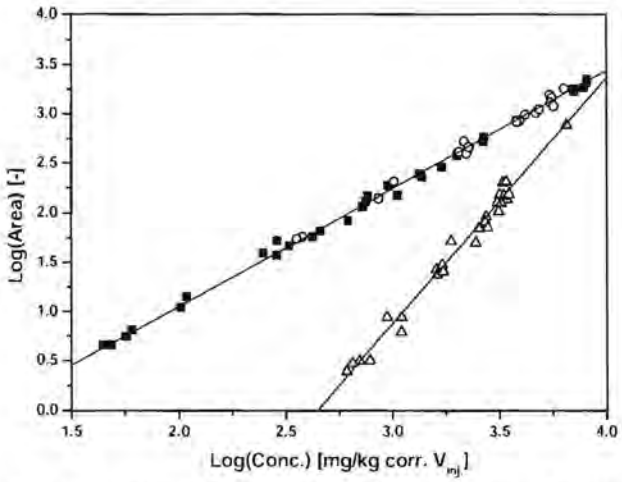

Figure 3-7: *Evolution of Log(Area) as a function of Log(concentration) based on the detector constants from Table 3-2. ■: linear hydroxy-ester species; ○: linear hydroxy-acid species; △: cyclic polymer species.*

Figure 3-7 shows the calibration lines for linear and cyclic polymer species under the assumption that the ELSD-detector cannot distinguish between linear hydroxy-acid species and linear hydroxy-ester species. These calibration lines have been applied for quantification of the reaction samples.

3.4.3. Quantification of the reaction samples

As a calibration line is obtained, this can be applied to quantify the concentration of the individual polymer species in the reaction samples of entries **1-6**. These samples were taken from e-ROP of ε-CL with different reaction conditions, as elaborated on before (section 3-3). In Table 3-3, the obtained concentration, recovery, and presence are listed for the reaction samples. The recovery, i.e. the ratio of the polymer concentration that was detected and the polymer concentration that was injected, in samples taken at the early stages of the polymerization (5-30 mins) is often found to be too low. This might be induced, as especially

initial reaction samples can be very sensitive towards differences in the exact polymer concentration (low polymer concentration). In addition, it must be noted that data obtained from ELSD are based on mass and do not provide information on the actual incorporation of nucleophile, as molecular weights may differ. In order to correct for this, a two-dimensional analysis technique should be considered, in which first separation on the basis of end-group is performed using LCCC. Subsequently, the molecular weight distribution of each polymer species can be obtained by MALDI-ToF-MS and/or SEC. However, the implementation of such a system is very complex and time-consuming for this dataset. Still, trends have become visible, and will be discussed in the Section 3-6.

Table 3-3: *Overview of the LCCC results for the reaction samples of entries **1-6**.*

Entry	Reaction time [mins]	M_n [g/mol]	[Hydroxy acid] [µg/g]	[Hydroxy Ester] [µg/g]	[Cyclic] [µg/g]	Recovery [-]	Hydroxy acid [w/w-%]	Hydroxy ester [w/w-%]	Cyclic [w/w-%]
1	5	970	950	580	755	0.20	42	25	33
	15	1390	702	661	935	0.84	31	29	41
	30	1750	617	405	706	0.38	36	23	41
	60	2490	1459	2087	1643	0.64	28	40	32
	120	4450	1728	2884	2308	0.91	25	42	33
2	5	1140	2188	183	< 1	0.28	92	8	< 1%
	15	1940	1427	315	< 1	0.19	82	18	< 1%
	30	3140	691	1922	621	0.89	21	59	19
	60	3480	832	3035	836	0.94	18	65	18
	120	n.d.	-	-	-	-	-	-	-
3	5	950	343	144	602	0.16	32	13	55
	15	1090	760	2023	3363	0.32	12	33	55
	30	1210	961	3720	5441	0.68	9	37	54
	60	1290	177	1137	2757	0.93	4	28	68
	120	1510	233	1852	4184	1.09	4	30	67
4	5	1190	3220	1680	1447	0.93	51	26	23
	15	2240	1933	161	1169	0.45	45	27	27
	30	3470	2477	2796	1844	0.86	35	39	26
	60	4930	2737	3886	1776	0.99	33	46	21
	120	6070	2103	4796	2202	1.00	23	53	24
5	5	1080	2933	< 1	3542	0.50	45	< 1%	55
	15	1260	3581	58	2880	0.71	55	1	44
	30	1620	5005	165	2598	1.11	64	2	33
	60	2130	1702	197	1368	0.57	52	6	42
	120	3200	3185	1224	1882	0.88	51	19	30
6	5	1060	1315	876	848	0.63	43	29	28
	15	1470	2074	1051	917	0.83	51	26	23
	30	2000	904	925	1368	1.01	28	29	43
	60	3230	1147	3023	2174	1.06	18	48	34
	120	5010	487	4147	2673	1.06	7	57	37

n.d. : not determined as the viscosity of the bulk system (entry **2**) was too high to take a sample.

3.5. *Evaluation of quantification by different techniques*

So far, two techniques have been discussed that provide information on the presence of different polymer species, namely LCCC (Table 3-3) and ^{1}H-NMR spectroscopy after end-group modification with oxalylchloride (Table 3-1). It has already been discussed that also MALDI-ToF-MS could be utilized to determine the presence per polymer species, if polymer standards would be available and an internal standard would be used.[6-9] When looking at a typical MALDI-ToF-MS spectrum of PCL synthesized by e-ROP (entry **2** in Table 3-1), it can be observed that the three distributions are present (Figure 3-8A/A'). Generally, cyclic polymer species are observed in the low-molecular weight region, and linear hydroxy-ester species are observed at higher molecular weights. Typically, linear hydroxy-acid species have very low intensities in MALDI-ToF-MS and seem to be underestimated, when compared to the hydroxy-ester species. This pattern is more clearly depicted in the spectrum of PCL that was initiated by water only, entry **Y** (Figure 3-8B). Even at higher molecular weights (2500-3000 g/mol), a region in which cyclic species are normally suppressed or not present[28], linear hydroxy-acid species are hardly detectable (Figure 3-8B'), whereas according to ^{1}H-NMR spectroscopy, the M_n of this polymer is significantly higher (5600 g/mol). Optimization of the matrix material and ionization agent could be considered to improve the analysis of polymer chains with carboxylic acid end-groups, however this was not applied. Additionally, it would be important to have polymer standards available for all three species individually, preferably in different molecular weights that can be used in the quantification of the different species using MALDI-ToF-MS.

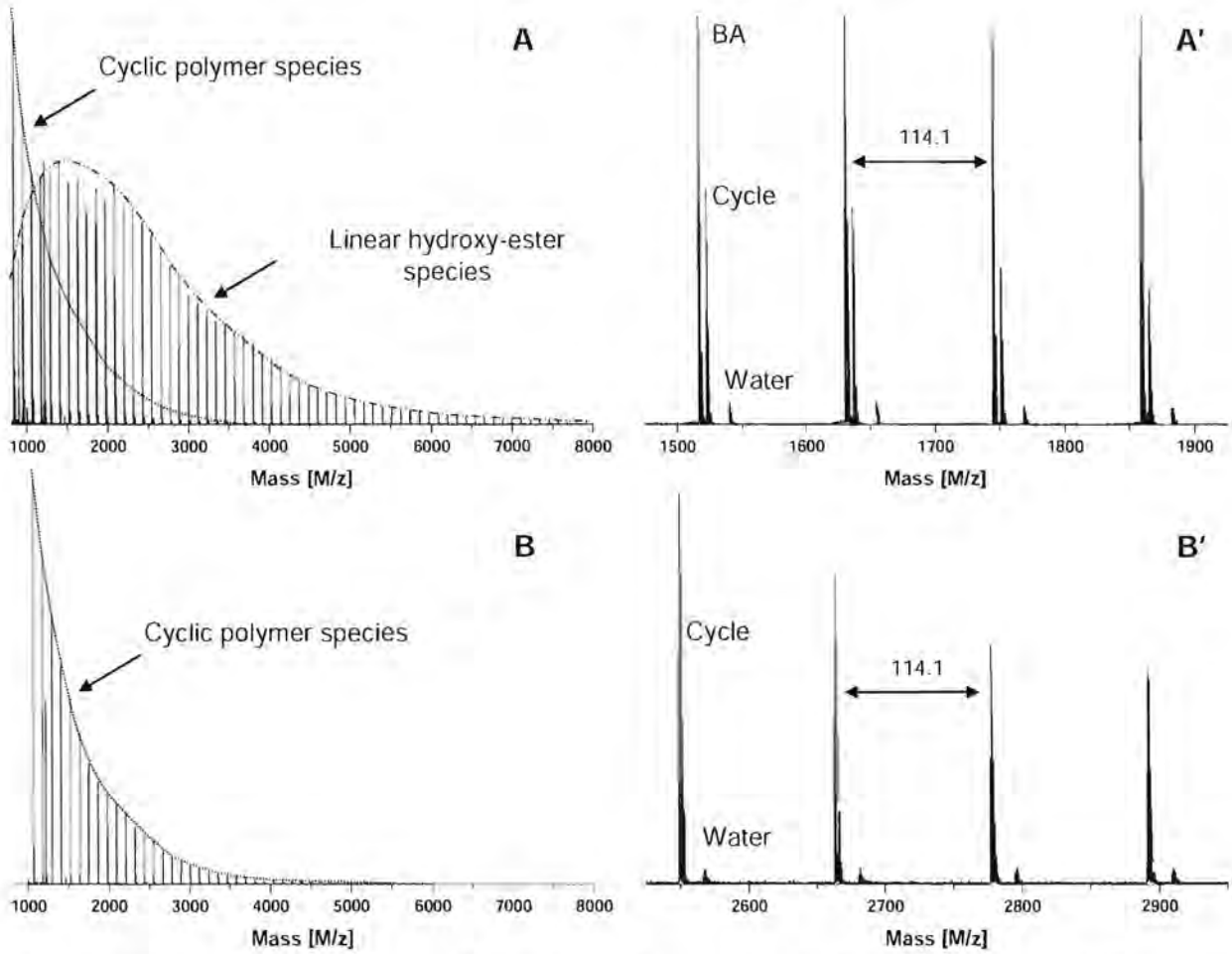

Figure 3-8: *MALDI-ToF-MS traces of PCL obtained from* **A**, **A'**: *entry 2 - bulk conditions with BA as initiator; after 60 mins;* **B**, **B'**: *entry* ***Y*** *(water as initiator; final polymer); the intensity of the highest peak in each spectrum was set to 100%. Lines are added to better visualize the different distributions.*

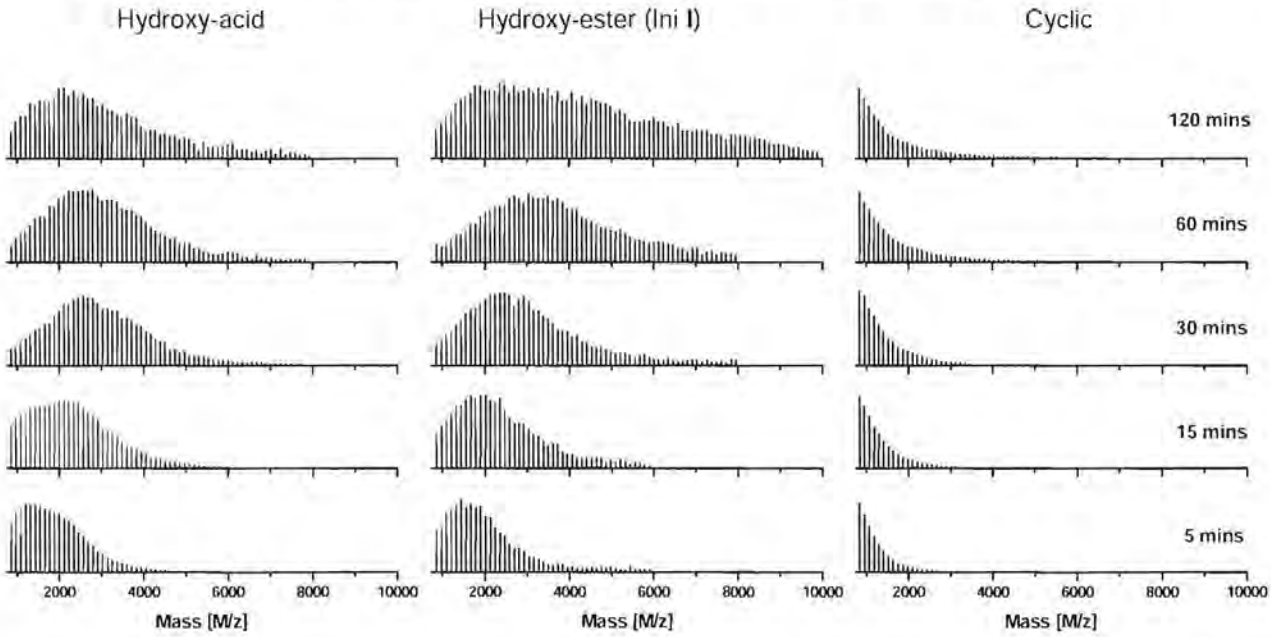

Figure 3-9: *Evolution of molecular weight for all specific polymer species during the reaction of entry 5, determined with MALDI-ToF-MS;* ***Left****: hydroxy-acid species;* ***Centre****: hydroxy-ester species from initiator* ***I****;* ***Right****: cyclic species. Numbers at the right bar represent reaction times in mins.*

An advantage of MALDI-ToF-MS for quantitative determination would be the ability to directly provide detailed information on the molecular weight distribution per individual polymer species (Figure 3-9 and 3-10). In Figure 3-9, the evolution of the molecular weight is depicted per polymer species during the reaction of entry **5** (bifunctional initiator **I**). It can be observed that the molecular weight increases during the reaction, for all three species. Moreover, it can be observed that the molecular weight distributions of both linear polymer species seem to broaden during the reaction. This is a clear effect of transesterification reactions. Cyclic species are predominantly observed at lower molecular weights, as was already concluded previously. It is still unclear, whether these species are original cyclic species in the polymer, or that part of these species are formed as an artifact in MALDI-ToF-MS, e.g. by cleavage of water.

Figure 3-10 shows the M_n and polydispersity indices of the final polymers of entries **1-6** depicted for the three individual species per entry. It can be observed that the polydispersity of the cyclic and linear hydroxy-ester species are in a similar range, whereas the polydispersity indices of hydroxy-acid species are significantly higher for most entries. This discrepancy is probably related to the low intensity of the signals of the hydroxy-acid species in MALDI-ToF-MS, resulting in inaccurate values when further applied. Additionally, it can be observed that the number average molecular weight, M_n, of cyclic polymer species is significantly lower than the M_n of both linear polymer species, and that the M_n of hydroxy-acid species is generally higher than that of hydroxy-ester species. Nevertheless, it cannot be concluded directly from MALDI-ToF-MS alone that cyclic polymer species are only formed with low molecular weight, as they may have been formed in the MALDI-ToF-MS analysis.

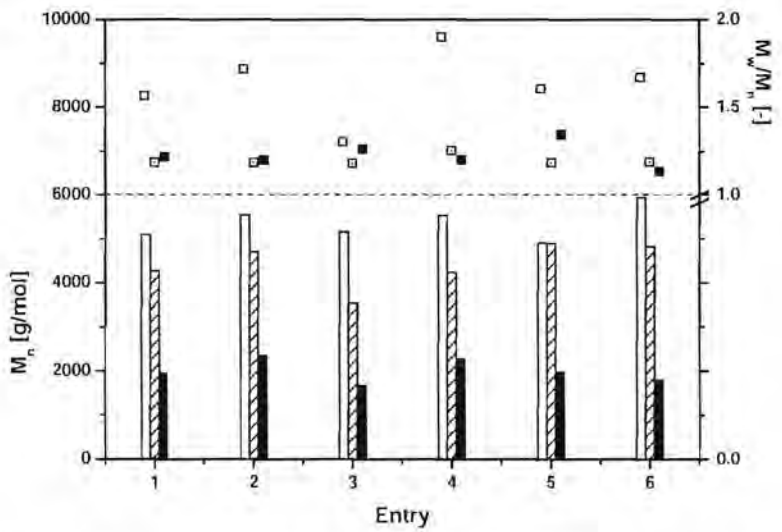

Figure 3-10: *Molecular weight (distributions) for all specific polymer species per entry, determined on final polymer with MALDI-ToF-MS; Open bars/squares represent linear hydroxy-acid species; patterned bars and squares represent linear hydroxy-ester species; solid bars/squares represent cyclic polymer species.*

A comparison can now be made between the detection of the individual polymer species in the final polymer determined by MALDI-ToF-MS, LCCC, and ^{1}H-NMR using oxalyl chloride. As ^{1}H-NMR spectroscopy and MALDI-ToF-MS detect the amount of polymer chains, the result will be expressed in molar ratios, whereas LCCC-ELSD detects concentrations, thus, a mass ratio will be obtained. Results from MALDI-ToF-MS can be converted into mass ratios by considering the product of the intensity and the corresponding molecular weight. Hence, a comparison can be made between MALDI-ToF-MS and LCCC on mass ratio, and another comparison between MALDI-ToF-MS and ^{1}H-NMR spectroscopy on molar ratio. In Table 3-4, the presence (in w/w-%) of linear hydroxy-acid species with respect to the total amount of linear polymer species in the final polymers (entries **1-6**) is listed, determined by LCCC and MALDI-ToF-MS (on mass basis). Additionally, the presence of linear hydroxy-acid species with respect to the total amount of linear polymer species in the final polymers (entries **1-6**) is given. From both ^{1}H-NMR spectroscopy and LCCC, it can be concluded that MALDI-ToF-MS underestimates the presence of hydroxy-acid species (Table 3-4).

In Figure 3-11, the presence of all three species determined with MALDI-ToF-MS (top) and LCCC (bottom) are depicted (mass ratios). Again, it can be observed that linear hydroxy-acid species are underestimated by MALDI-ToF-MS. Moreover, also cyclic polymer species are underestimated by MALDI-ToF-MS when compared with LCCC.

Table 3-4: *Presence of linear hydroxy-acid species related to the total amount of linear species determined by MALDI-ToF-MS and LCCC, and MALDI-ToF-MS and ^{1}H-NMR spectroscopy after end-group modification with oxalyl chloride. Values are discussed in section 3-6.*

Entry	LCCC [w/w-%]	MALDI-ToF-MS [w/w-%]	MALDI-ToF-MS [mol-%]	^{1}H-NMR spectroscopy [mol-%]
1	7	2	7	33
2	11	2	3	34
3	2	2	2	48
4	34	9	6	36
5	41	10	18	59
6	8	10	6	20

In summary, evaluation of the applied techniques revealed that ^{1}H-NMR spectroscopy functions properly as a technique to determine the ratio of linear species only. As this technique cannot provide any information on the amount of cyclic polymer that is formed during the reaction, it is not a complete analysis technique for the PCL obtained in e-ROP. MALDI-ToF-MS has proven its capacity to provide a lot of data at once, but is only to be used qualitatively, when studying the formation of different polymer species. This is caused by mass discrimination and the specific preference of some polymer end-groups over others (e.g. linear hydroxy-ester species over linear hydroxy-acid species). It is doubtful, whether

this technique could be available for a quantitative study towards the obtained polymer species, as mass discrimination is believed to be inherent to MALDI-ToF-MS.[29-31] LCCC is a characterization technique that comes with some challenges as obtaining critical conditions is a delicate process. Moreover, quantification of reaction samples can be particularly complicated, when monomer is still available. Small molecules are not detected by the ELSD, creating a difference between the injected and the obtained polymer concentration (i.e. recovery). Nevertheless, it must be stated here that LCCC is the only characterization technique that can directly provide quantitative information on all three polymer species. Still, the issue of differences in sensitivity of the ELSD towards cyclic and linear polymer species might be reconsidered, as a consequence of the data shown in Figure 3-6. The observed presences of cyclic polymer species (Table 3-3, Figure 3-11) represent maximum values. When equal sensitivity is assumed, the presence of cyclic polymer species would be lower.

In Figure 3-12**A** and **B**, LCCC-chromatograms are depicted in line with SEC-chromatograms from identical samples. Figure 3.12**A** shows the evolution of the molecular weight distribution and the formation of species of reaction samples of entry **3** (diluted conditions). In the LCCC-chromatograms it can be observed that in the first 5 mins of e-ROP, benzyl alcohol is already introduced to the polymer (linear hydroxy-ester species). However, most linear species (w/w-%) are linear hydroxy-acid species, i.e. polymer chains that are initiated by water. After 15 minutes, linear hydroxy-ester species (started from BA) are the predominant linear species. Over time, the concentration of linear hydroxy-acid species seems to decrease. However, in the final polymer 48% water-initiation was detected on molar basis (^{1}H-NMR spectroscopy; Table 3-1). Additionally, it is observed that in dilute conditions (entry **3**, Figure 3.12**A**), cyclic polymer species are present to a large extent during all stages of the reaction (up to 70 w/w-%). When the evolution in the presence of the three species is combined with the SEC-chromatograms, it can be observed that the molecular weight increases. Although after 30 mins a peak seems to be formed, the chromatograms reveal a significant amount of 'non-growing' polymer (at low molecular weight), probably due to transesterification reactions. Therefore, a considerable increase of the polydispersity index is observed (5 mins: PDI = 1.68; 120 mins: PDI = 2.31).

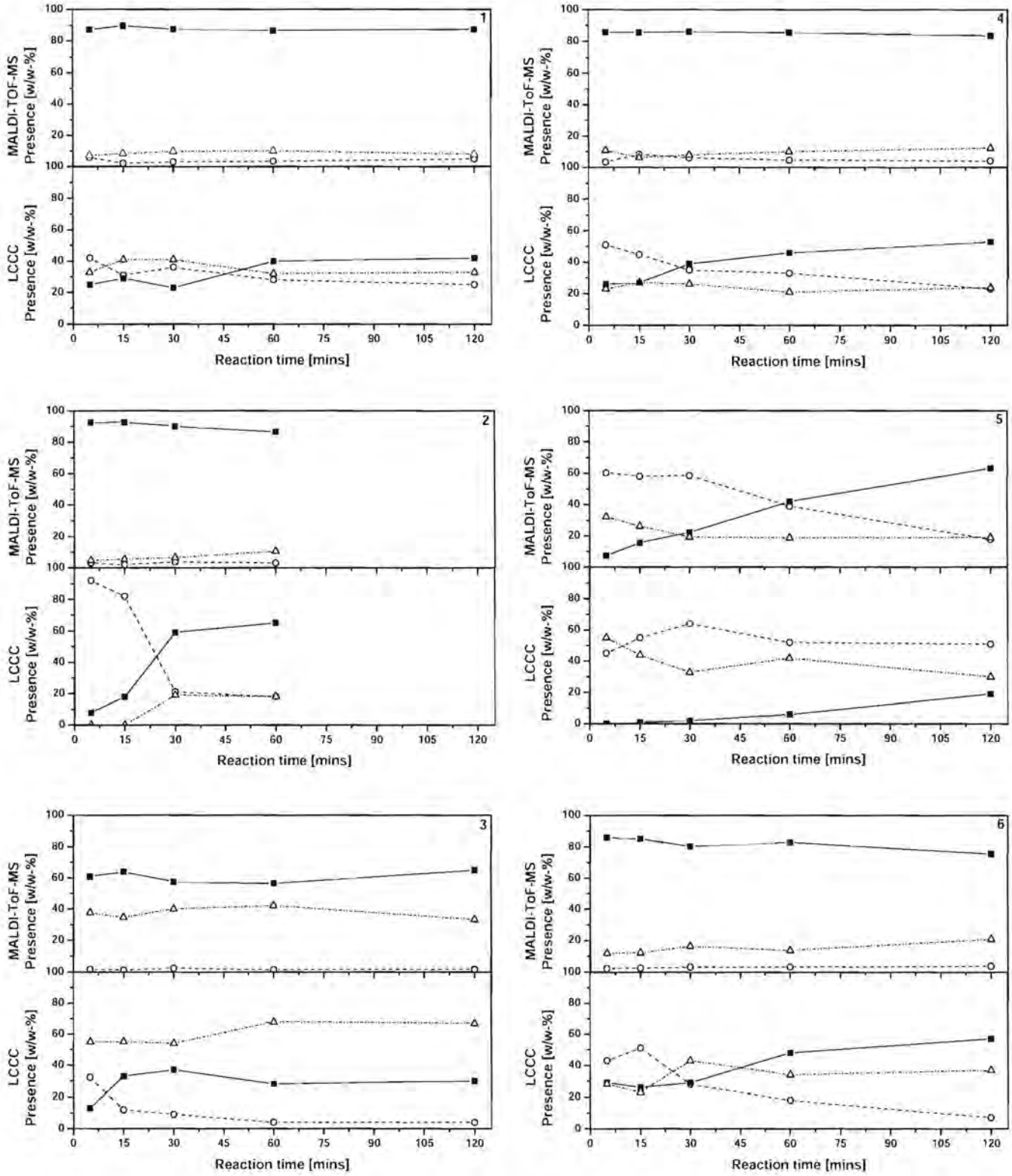

Figure 3-11: *Evolution of the presence per polymer species (w/w-%) as a function of reaction time, determined by MALDI-ToF-MS (top) and LCCC (bottom); ■: linear hydroxy-ester species, ○: linear hydroxy-acid, △: cyclic polymer; numbers in top right corner refer to entry numbers*

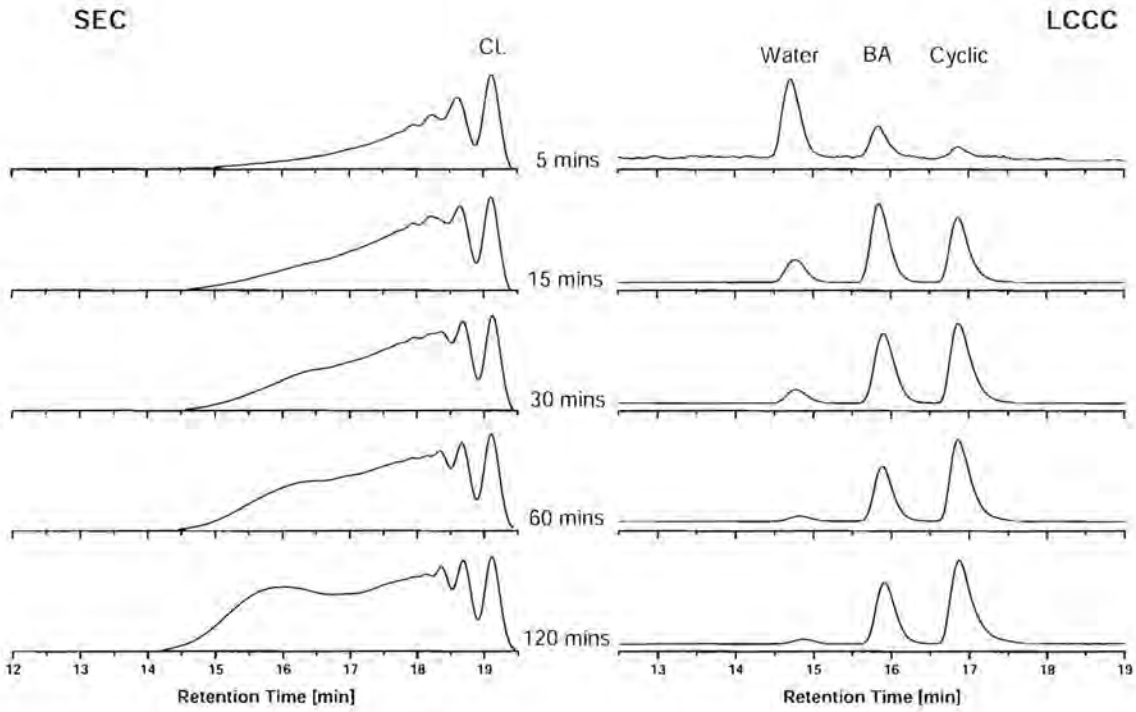

Figure 3-12A: *SEC- and LCCC-chromatograms as a function of reaction time for entry 3 (diluted conditions and benzyl alcohol as initiator).*

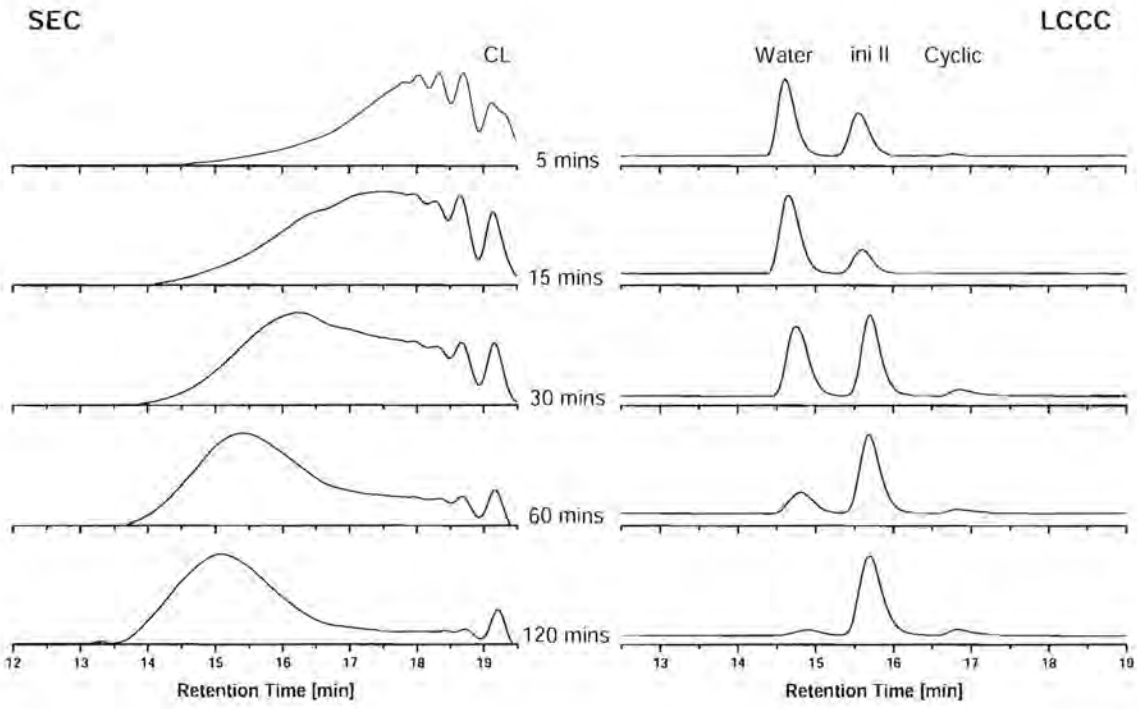

Figure 3-12B: *SEC- and LCCC-chromatograms as a function of reaction time for entry **6** (bifunctional initiator **II**).*

Figure 3.12**B** shows the LCCC-chromatograms and corresponding SEC-chromatograms of the reaction samples of entry **6** (initiated by bifunctional initiator **II**). In the LCCC-chromatograms, the evolution of linear hydroxy-acid species can be demonstrated during the reaction. Although it is assumed that initiator **II** is a good initiator in the e-ROP of ε-CL, it is observed that after 5 minutes, the majority of species is initiated by water. During the reaction, the presence of linear hydroxy-acid species is reduced in favour of linear hydroxy-ester species and cyclic polymer species. The evolution of the molecular weight distribution in this entry **6**, is different from entry **3** (Figure 3-12**A**). In entry **6**, a peak is formed in 15 mins and only a minor fraction of the polymer seems to be 'non-growing'. Hence, higher molecular weight polymers can be achieved.

3.6. Effects of reaction conditions on e-ROP of ε-CL

In this section, the results of all characterization techniques that have been applied on the polymers will be combined to obtain detailed information on e-ROP of ε-CL at different conditions. In order to distinguish between the different reaction conditions, the results will be discussed individually with respect to the effect of monomer concentration, amount of water present, and type of initiator.

3.6.1. Monomer concentration

The effect of monomer concentration on the formation of linear and cyclic polymer species can be revealed by comparing entries **1-3**. As discussed earlier in Chapter 2, intramolecular transesterification reactions are expected to be more pronounced in more diluted systems.[1, 2, 32, 33] Transesterification reactions will generally lead to (i) broadening of the molecular weight distribution due to chain transfer, and (ii) a decrease of the amount of linear hydroxy-ester polymer species, as a result of the cycle-formation. As an initiator normally is added to introduce a specific functionality into the polymer, a transesterification reaction can be regarded as an undesired side-reaction. Regarding the first effect, i.e. broadening of the molecular weight distribution, Table 3-1 shows that entries **2** and **3** have slightly narrower molecular weight distributions than the reference system (entry **1**). Nevertheless, it was observed that the polydispersity increases differently in a diluted system (entry **3**) than in more concentrated systems at higher conversions (Figure 3-12**A**; Chapter 2: Figure 2-5). As the conversion in this system (entry **3**) was only 59%, its molecular weight distribution is expected to continue broadening. In more concentrated systems, the polydispersity is found to stabilize during the reaction. Regarding the second effect, i.e. cyclic polymer formation, both LCCC and MALDI-ToF-MS show that in a diluted system (entry **3**) up to 70 w/w-% of cyclic polymer species are formed, whereas in a bulk system (entry **2**), cyclic polymer formation can

be reduced to 20 w/w-% (Figure 3-11). In the latter entry, the presence of cyclic polymer species could also explain the low M_n for the obtained polymer (1300 g/mol).

Typically, entry **5** reveals a higher initial amount of cyclic polymer species (LCCC). This formation might be due to the low nucleophile concentration in the initial stages of the reaction, as bifunctional initiator **I** does not seem to act as a competitive nucleophile towards water. When the initiator is incorporated during the reaction, the amount of cyclic polymer species seems to decrease. Hence, dilution not only affects the polymerization by a reduced monomer concentration, but possibly also by a reduced initiator concentration.

Generally, it can be concluded that in (enzymatic) ring-opening polymerization, a diluted system promotes the formation of cyclic polymer structures, consequently limiting the obtained molecular weight of the polymer, and broadening the molecular weight distribution, as chain transfer is favoured.

3.6.2. Water

In all polymerizations, it was observed by LCCC that predominantly linear hydroxy-acid species are formed in the initial stage of the reaction (Figure 3-11 and 3-12). A typical scissor-like pattern is observed when the presences of the two linear polymer species during the reaction are compared (Figure 3-11). This effect seems to be stronger in a bulk system, possibly as mass transport of initiator molecules to the active site is initially slower. As a result, water is the primary nucleophile to start the enzymatic polymerization. This conclusion can be explained when it is considered that traces of water are still present in the enzyme and the support, even after drying, whereas the initiator first has to diffuse into the enzyme. During the polymerization, also polymer chains bearing initiator functionality are formed, either by initiation or by incorporation as a result of transesterification.

In Table 3-4, the presence of linear hydroxy-acid species with respect to the total amount of linear polymer species is listed, determined by all three techniques. It was observed with ^{1}H-NMR spectroscopy, that all polymers contain substantial amounts of water-initiated polymer chains (on molar basis). By LCCC, the presence of linear hydroxy-acid species with respect to the total amount of linear polymer species can also be determined, but only on mass basis. Typically, these results show lower weight amounts of linear hydroxy-acid species than the molar amounts obtained from ^{1}H-NMR spectroscopy. If both techniques produce accurate results, this would be an indication that the molecular weight of the hydroxy-acid polymer species is lower than that of the hydroxy-ester species.

3.6.3. Initiator

It was mentioned previously, that water is observed to start initiation of the polymer chains, as initially it is located closer to the active site of the enzyme. With LCCC, it is also

observed that benzyl alcohol is built into the polymer chains rather quickly (Figure 3-12A). It is assumed that in the case of benzyl alcohol the incorporation of initiator primarily occurs as a result of initiation rather than transesterification. This assumption is supported by MALDI-ToF-MS, which shows an increase in molecular weight of the hydroxy-ester species during the reaction, starting at low molecular weights. The same pattern was observed when bifunctional initiator **II** was applied.

However, MALDI-ToF-MS revealed a distinct broadening of the molecular weight distribution of hydroxy-ester species when bifunctional initiator **I** was applied. When this observation is combined with the result of the initiator conversion during the reaction (Table 3-1), it can be concluded that bifunctional initiator **I** is mainly incorporated via transesterification. This difference in initiating behavior may have been caused by the size of the initiator molecule, as it is a large molecule that could suffer from steric hindrance in the active site of the enzyme. Additionally, initiator **I** contains a chiral centre adjacent to the primary alcohol group, which might explain the initiation conversion of only 50%. Hence, it can be concluded, that specific end-functionality can be introduced to a polymer, by an initiator bearing this functionality, provided that the size and shape of this initiator allows easy entrance to the active site of the enzyme during the polymerization.

3.7. Conclusions

In this chapter, the influence of reaction conditions on enzymatic ring-opening polymerization (e-ROP) of ε-caprolactone (ε-CL) was further investigated using polymer characterization techniques such as MALDI-ToF-MS and ^{1}H-NMR spectroscopy, as well as the less common technique of liquid chromatography under critical conditions (LCCC). This latter technique provides substantial advantages over common characterization methods, as all polymer species can be taken into account. Careful adjustment of the critical conditions allow for good separation of the polymer species independent of molecular weight. By choosing an appropriate detector (e.g. ELSD), quantification of the polymer species can be performed, providing accurate information on the obtained polymer species. When applying an ELSD-detector, the polymer sample should not contain monomer or dimer species, as these species cannot be detected by ELSD, which finally results in too low recoveries.

In this investigation, e-ROP of ε-CL was performed under different reaction conditions in order to observe and quantify the influence of monomer concentration, the influence of water, and the influence of the applied initiator on the obtained polymer species. By careful analysis of samples taken during the polymerization, new insights were obtained in the process of e-ROP. It was observed that water-initiated polymer chains (linear hydroxy acid species) are the predominant species in the initial stages of the reaction. Hence, it is believed that water acts as

initial nucleophile in the early stages of the polymerization, creating linear hydroxy-acid species. If untreated enzyme is used, up to 40 w/w-% of hydroxy-acid species can be formed after 120 mins of polymerization. When aiming for end-functionalized polymer, the size and type of the nucleophile that is used as initiator determine its efficiency. Bifunctional initiator **II** was observed to act as good nucleophile (comparable to benzyl alcohol), whereas bifunctional initiator **I** was found to act as a poor nucleophile. Monomer concentration was found to be the primary tool to reduce the formation of cyclic polymer species. Polymerization under bulk conditions suppresses intramolecular transesterification reactions (<20 w/w-% cyclic species), whereas in very diluted systems (0.7 M), up to 70 w/w-% of cyclic species can be observed.

The maximum presence of linear end-functionalized polymer was obtained by applying bulk conditions, and by the use of bifunctional initiator **II** as nucleophile, and was ~60 w/w-% in this investigation. It is expected, that combining both conditions, i.e. bulk conditions and initiator **II** in combination with properly treated enzyme, could result in predominantly end-functionalized polymer (>90%).

3.8. *Experimental section*

Materials

All chemicals were purchased from Aldrich and used without further purification unless otherwise noted. ε-Caprolactone (ε-CL) was distilled over CaH_2 and stored on molecular sieves. Toluene was dried over aluminum oxide and stored on molecular sieves. Molecular sieves (3Å) were dried in an oven at 420 °C before use. Novozym 435™ was obtained from Novozymes A/S and stored over P_2O_5 in a desiccator. The enzyme was then dried before use according to procedure **7** as described in Chapter 2. Bifunctional initiators **I** and **II** were synthesized and characterized according to literature procedures.[34, 35]

Methods

Size exclusion chromatography (SEC) was performed on a Waters Model 510 pump and Waters 712 WISP, using PL-gel mix D columns (300 x 7.5 mm, Polymer Laboratories) at 40 °C. THF was used as eluent with a flow rate of 1.0 mL/min. All samples were diluted to 1.0 mg/mL and filtered using 0.2 μm syringe filters. All samples were calibrated using universal calibration: K = 0.00109 dL/g and a = 0.6.[36] ^{1}H-NMR spectroscopy was performed using a VARIAN 400 MHz NMR at 20 °C. Samples were dissolved in $CDCl_3$ to a concentration of ~30 mg/mL. Data were acquired using VNMR-software. MALDI-ToF-MS analysis was carried out on a Voyager DE-STR from Applied Biosystems using trans-2-[3-(4-tert-

butylphenyl)-2-methyl-2-propenylidene]-malononitrile (DCTB) as matrix material. All spectra were recorded in the reflector mode. Samples were prepared using 1 mg/mL of polymer in THF. The ratio of polymer sample to matrix was 1 : 5 (w/w-%). Karl-Fischer Coulometry was performed on a Mettler Toledo Titrator DL39 with APURA CombiCoulomat fritless electrolyte.

All LCCC-experiments were conducted on an Agilent 1100, equipped with a quaternary pump, degasser, autosampler, column oven, and a diode-array detector (DAD) with 10-mm cell (Agilent, Waldbronn, Germany) at 40 °C. The mobile phase, 17.5 w/w-% ultra-pure water (1.5 vol-% formic acid) and 82.5 w/w-% THF, was mixed and pumped with a flow rate of 0.5 ml/min. Four Nucleosil 120-5 (Machery-Nagel) C18-columns in series (250x4 mm) were used to establish the critical separation. The injection volume was 5 µL. Detection with a Sedex 75 ELSD system (Sedere, Vitry/Seine, France) was performed with 3.6 bar air pressure at the nebulizer and a drift tube temperature at 35 °C. The detector signal was collected with an Atlas version 8.1 data management system (Thermo Electron Cooperation, Manchester, UK).

Enzymatic Polymerization of ε-Caprolactone (ε-CL)

Different polymerizations were conducted using reference conditions as follows. In a 25 mL round bottom flask, equipped with a magnetic stirrer bar, 250 mg Novozym 435 was dried in a vacuum oven at 50 °C for 16 hours (procedure **7** in Chapter 2). The vacuum was released by N_2 and dried molecular sieves were added to the flask. Subsequently, the flask was placed in an oil bath at 60 °C. The polymerization was started by the addition of a stock solution of ε-caprolactone and benzyl alcohol in toluene (5.15 g; 4.10 M and 56.5 mg; 47 mM, respectively). Immediately after the start of the reaction, a sample was withdrawn from the reaction mixture and analyzed using Karl-Fischer Coulometry. At specified time intervals further samples (~0.15 mL) were withdrawn from the reaction mixture with a syringe for analysis. After 120 mins , the reaction was stopped by removing Novozym 435 by filtration using dichloromethane as solvent. The polymer was dried under vacuum at 40 °C.

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4

Chemoenzymatic Synthesis of Block Copolymers

Abstract

The applicability of enzymatic ring-opening polymerization (e-ROP) in combination with radical polymerization techniques such as Atom Transfer Radical Polymerization (ATRP) was investigated. Block copolymers comprising ε-caprolactone (ε-CL) and methyl methacrylate (MMA) as monomers were synthesized from a bifunctional initiator via two-pot and one-pot approaches. The choice of the initiator, monomer and ATRP-catalyst were found to be of crucial importance in order to avoid the formation of side-products and to retain enzyme activity. The block copolymers and intermediate products were characterized using ^{1}H-NMR, SEC, GPEC and MALDI-ToF-MS to obtain accurate information on the formed polymer species. Via a two-pot approach, block copolymer was easily obtained in relatively pure form when e-ROP was conducted first. In a consecutive one-pot approach, MMA was observed to be activated by Novozym 435, forming methanol and methacrylate end-capped PCL-chains. Using an 'inert' methacrylate such as t-BMA resulted in a block copolymer using this consecutive approach. In an ultimate cascade approach (simultaneous one-pot), poorly-defined block copolymers were synthesized, due to single initiation of the ATRP-reaction, thereby disallowing any enzymatic polymerization on the same polymer chain.

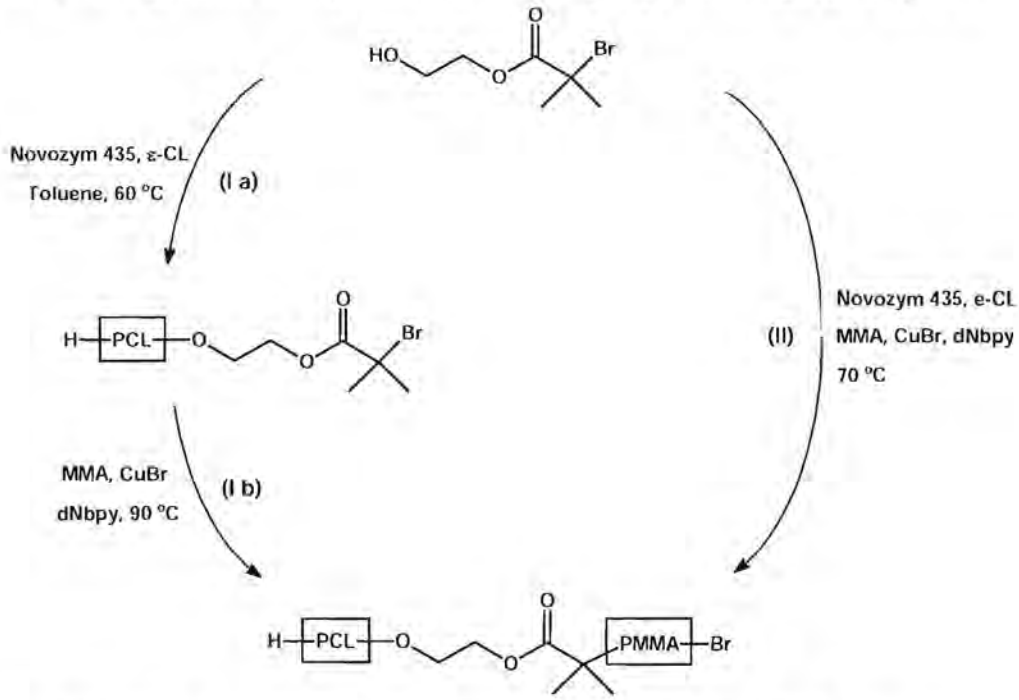
4.1. Introduction

The design of novel materials often requires the development of new types of polymers. As novel monomers are often expensive and not directly available, new synthetic pathways and polymer architectures are investigated to obtain well-defined polymers. Over the past decades, an increased interest has arisen in the use of block and graft copolymers, of which properties can be controlled by the chemical composition. The application of these types of materials can be found in compatibilizers, impact modifiers, coating materials, drug delivery systems, and information storage systems.[1] Different pathways for the synthesis of block copolymers are available: the most direct approach is a coupling reaction of two homopolymers. The main disadvantage of this approach is that the used homopolymers are limited to low molecular weights, since the probability of reactions at the chain ends leading to block copolymer formation is significantly reduced with increasing molecular weight.[2] A second approach is the synthesis by sequential addition of monomers to a living polymerization system. Via this approach, block copolymers can only be synthesized via one and the same technique and this technique is restricted by relative monomer reactivities.[3] A third route to block copolymers is the transformation approach, in which different polymerization mechanisms can be sequentially combined, allowing the incorporation of different types of monomers. In a first step, a prepolymer is synthesized and terminated. This terminated polymer species can be modified using the appropriate functionality for the consequent polymerization technique. Of course, this approach requires an intermediate protection and transformation step, which makes the synthesis more elaborate.[4] An approach that can avoid these synthetic drawbacks is the use of a heterofunctional initiator, which contains two or more different initiation sites that are capable of initiating concurrent polymerization techniques independently. This approach avoids an intermediate transformation and protection step and hence provides the possibility to synthesize block copolymers via one-pot synthesis.[5, 6] A recent review article on the use of heterofunctional initiators in the synthesis of block copolymers has summarized the latest activities.[7] By choosing an appropriate initiator that is capable of initiating intrinsically different polymerization techniques, the latter route allows the polymer chemist to use enzymatic ring-opening polymerization (e-ROP) with controlled radical polymerization techniques such as atom transfer radical polymerization (ATRP) and nitroxide-mediated radical polymerization.[8, 9]

In polymer chemistry, the use of enzymes has several advantages over traditional catalysts[10, 11], nevertheless, the full exploitation of biocatalysis in polymer science will require the development of mutually compatible chemical and biocatalytic methods. Therefore, it is important to investigate the integration of biocatalytic and conventional polymer synthesis based on radical polymerization. The objective of the present investigation is the development of a chemoenzymatic cascade polymerization, i.e. combined catalytic reactions without

intermediate recovery steps, for the synthesis of block copolymers. It is believed that this new concept in polymer science might eventually lead to the development of new materials and sustainable technology, as enzymes offer the unique opportunity to perform and control regio- and enantioselective polymerizations.[12, 13] However, its versatility has to be proven first on a fundamental level.

As a model system, the synthesis of block copolymers by the combination of controlled radical polymerization and e-ROP was investigated. The synthesis of block copolymers was particularly suited as a proof of principle, since the marriage of two chemically and sometimes structurally different building blocks often provides a considerable synthetic challenge. The feasibility of chemoenzymatic cascade polymerization combining nitroxide-mediated radical polymerization and e-ROP has been reported by Van As et al.[9] This investigation showed that the compatibility of both techniques, and the fine tuning of the reaction rates, are of paramount importance in achieving high block copolymer yields.



Scheme 4-1: *Different approaches for chemoenzymatic polymerization; (I): Two-pot approach, and (II): Consecutive and simultaneous one-pot approach.*

In order to achieve complete control and understanding towards the compatibility of Novozym 435 with respect to other monomers and catalysts, a two-pot system was initially studied in which first enzymatic polymerization of ε-caprolactone (ε-CL) was performed from a bifunctional initiator, as depicted in Scheme 4-1 (**I a**). The knowledge obtained in Chapters 2 and 3 was applied to obtain linear poly(ε-caprolactone) (PCL) bearing only the specific functionality for consecutive controlled radical polymerization. The synthesized PCL was purified and used as macro-initiator in a successive ATRP of methyl methacrylate (MMA) to obtain poly(CL-*b*-MMA) (**I b**). The obtained block copolymer was characterized by size

exclusion chromatography (SEC), ^{1}H-NMR spectroscopy, and gradient polymer elution chromatography (GPEC) to prove block copolymer formation, rather than the formation of the two homopolymers. Subsequently, cascade block copolymer synthesis was investigated via a consecutive one-pot approach (**II**). In such 'semi-batch' systems, the e-ROP could only work, if the lipase was compatible with the metal-complex used in the ATRP of MMA. This compatibility was proven by performing e-ROP in the presence of both the metal halide and different ligands. Again, the obtained block copolymer species were analyzed extensively. Finally, cascade block copolymer synthesis was investigated via a simultaneous one-pot approach (**II**). In here, both polymerization techniques were performed at the same time, providing the ultimate proof for the compatibility of enzyme- and chemically catalyzed polymerization.

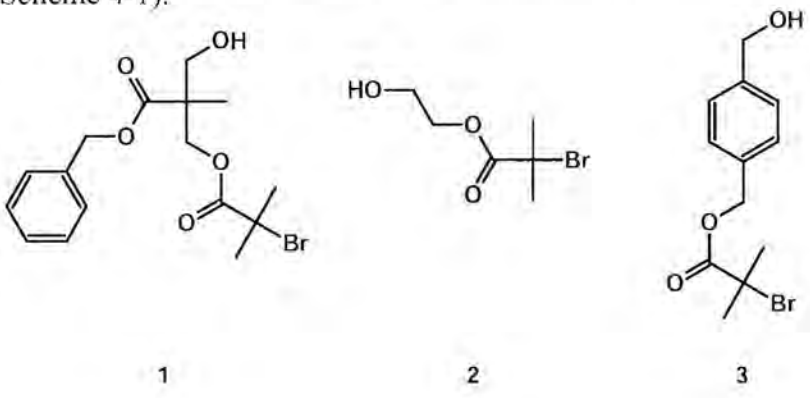
4.2. *Two-pot block copolymer synthesis*

As outlined in Scheme 4-1, the combination of ATRP and e-ROP for the synthesis of block copolymers can either be conducted in two consecutive steps, or in a one-pot cascade approach. The latter provides, without a doubt, the higher synthetic and analytical challenge. In order to obtain high block copolymer yields, it is of paramount importance to fine tune the reaction rates and to minimize side reactions leading to homopolymer formation. Therefore, the governing factors to minimize the extent of undesired side reactions were carefully investigated in order to obtain a maximum level of control over the polymerization and hence the polymer structure. Typical side-reactions (e.g. the formation of cyclic polymer and carboxylic acid end-groups) have been discussed in the previous two chapters. Only the use of a bifunctional initiator bearing an activated halide-functionality (e.g. a 'tertiary' bromide-group) for ATRP and a nucleophilic group (e.g. primary hydroxy-group) for e-ROP allows the synthesis of block copolymers without an intermediate transformation step.[7] The key to obtain high block copolymer yields is (i) high initiation efficiency from both initiator groups and (ii) initiation exclusively from the bifunctional initiator. Low initiator efficiency in either one of the two polymerizations or initiation by a species other than the bifunctional initiator inevitably results in homopolymer formation. Since the efficiency of ATRP is generally known to be high, we have primarily focused our effort on the optimization of the e-ROP, i.e., the conditions that lead to a high initiator efficiency in the enzymatic reaction.

4.2.1. Initiator kinetics

Three different bifunctional initiators (**1-3** in Figure 4-1) differing in size, were synthesized for block copolymer synthesis. Since all initiators possess a relatively bulky structure, due to the 'tertiary' bromine-functionality, first their feasibility in lipase catalyzed ε-CL

homopolymerization was investigated. The objective was to identify the structure with the highest initiation efficiency that leads to a high degree of end-functionalized PCL according to route **Ia** (see Scheme 4-1).



1 2 3

Figure 4-1: *Representation of bifunctional initiators 1, 2, and 3.*

Benzyl alcohol was chosen as a reference initiator because of its known high initiator efficiency in the e-ROP. All reactions were conducted at 60 °C for 60 mins in triplicate with Novozym 435 dried according to procedure **7** (Chapter 2). Figure 4-2 shows the consumption of the different initiators during the polymerization, determined by ^{1}H-NMR spectroscopy. Careful inspection of Figure 4-2A illustrates the rapid conversion of benzyl alcohol, being >90% in 15 mins. Similar results were found for initiator **2** and **3** (Figure 4-2B), the latter resembling the structure of benzyl alcohol. Both initiators are consumed at a similarly high rate. Initiator **1**, on the other hand, is a poor initiator in this polymerization, as evidenced from the incomplete initiator consumption after one hour, and the slower monomer consumption (Figure 4-3A). The observed inhibition period in the polymerization using **1** implies that small amounts of water, present in the enzyme, initiate the polymerization causing very slow monomer and initiator conversion. This is due to the low concentration of nucleophiles able to react with the enzyme-activated monomer species. These results further suggest that the incorporation of benzyl alcohol and bifunctional initiator **2** and **3** into the growing polymer chain is predominantly through initiation. In contrast, initiator **1** seems to be built into the chain by transesterification during the course of the reaction.

As a consequence, high end-group functionalization with **1** can only be achieved at longer reaction times like those applied for the synthesis of ATRP initiator end-capped PCL in an early report.[14] The behavior of initiator **1** was already discussed in Section 3.6.3, where it was concluded that this initiator is predominantly incorporated in the PCL-chain by transesterification, due to its bulky structure and/or the chiral centre adjacent to the nucleophilic group. Although difficult to predict, chirality in both the initiator and the monomer can have a dramatic influence on the kinetics of enzymatic polymerizations.[15]

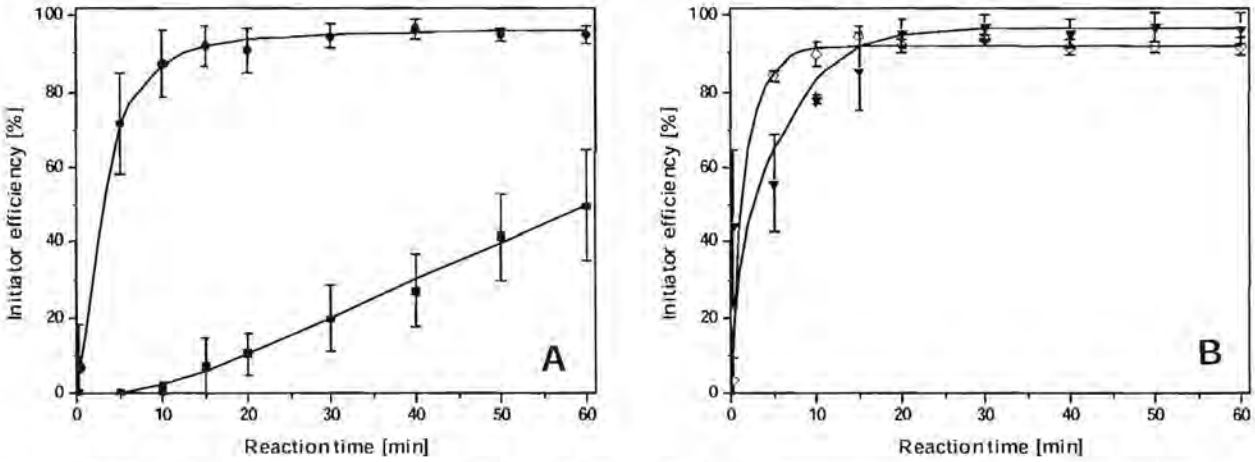


Figure 4-2: *Initiator conversion in e-ROP of ε-CL at 60 °C, determined by ^{1}H-NMR spectroscopy employing A: Benzyl alcohol (•) and initiator 1 (■); B: Initiator 2 (◊) and 3 (▼). Error margins are constructed from three parallel reactions and lines are drawn to guide the reader's eye.*

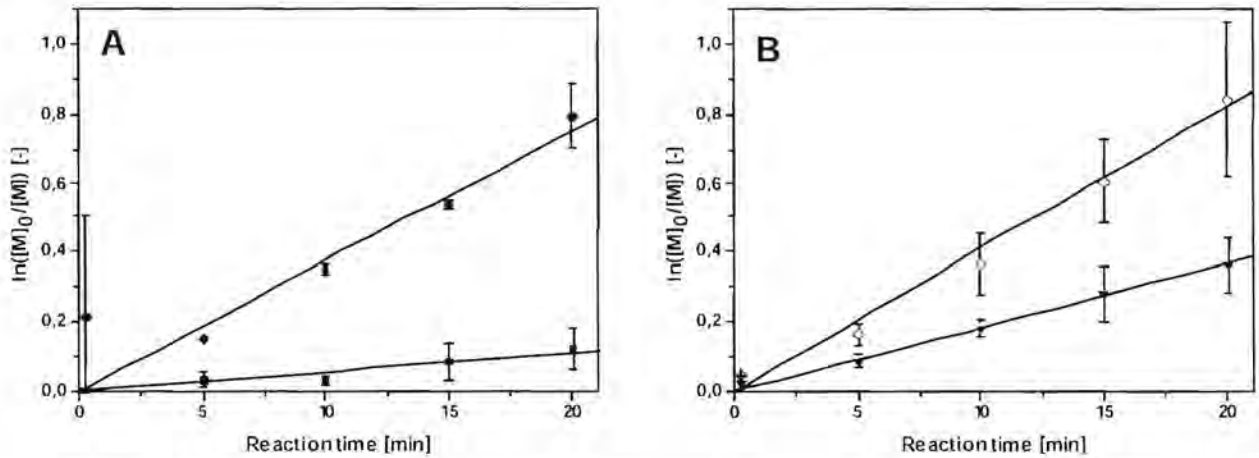


Figure 4-3: *Kinetic plots of monomer consumption as a function of time, determined by ^{1}H-NMR spectroscopy on samples withdrawn from e-ROP of ε-CL at 60 °C using different initiators: A: Benzyl alcohol (•) and Initiator 1 (■); B: Initiator 2 (◊) and 3 (▼). Error margins are constructed from three parallel reactions and lines are drawn to guide the reader's eye.*

The kinetics of the e-ROP were found to be first order with respect to monomer consumption (as depicted in Figure 4-3), regardless of the used bifunctional initiator, which agrees with previous literature reports.[16] Nevertheless, the rate of polymerization is supposed to be independent of the nucleophile (i.e. initiator), whereas a clear relationship is observed. Reason for this discrepancy is expected to be the actual low amount of water present in the reaction whilst performing the kinetic experiments. If the water concentration would be higher, slight differences in activity between the initiators might not be observed. The development of the molecular weight was followed by SEC on samples withdrawn at predetermined time intervals from the reaction mixture. In Figure 4-4 the evolution of the molecular weight distribution is depicted for a typical e-ROP of ε-CL using initiators **1** and **2**, respectively. Although the final products look similar, a different pattern can be observed at

the early stage of the polymerization. When initiator **1** was applied, initially, no reaction seemed to take place, and only after 10 mins polymer chains showed up. As only little initiator was consumed, water - although barely present – might have initiated the first chains, allowing some polymer formation. When initiator **2** is applied, monomer is immediately converted into polymer (as can be derived from ^{1}H-NMR spectroscopy and SEC). It becomes evident from these chromatograms that the polymerization proceeds with better control when using initiator **2**. Based on the optimized reaction conditions, resulting in a high degree of incorporation of the bifunctional initiator, PCL macro-initiators were synthesized using initiator **2**.

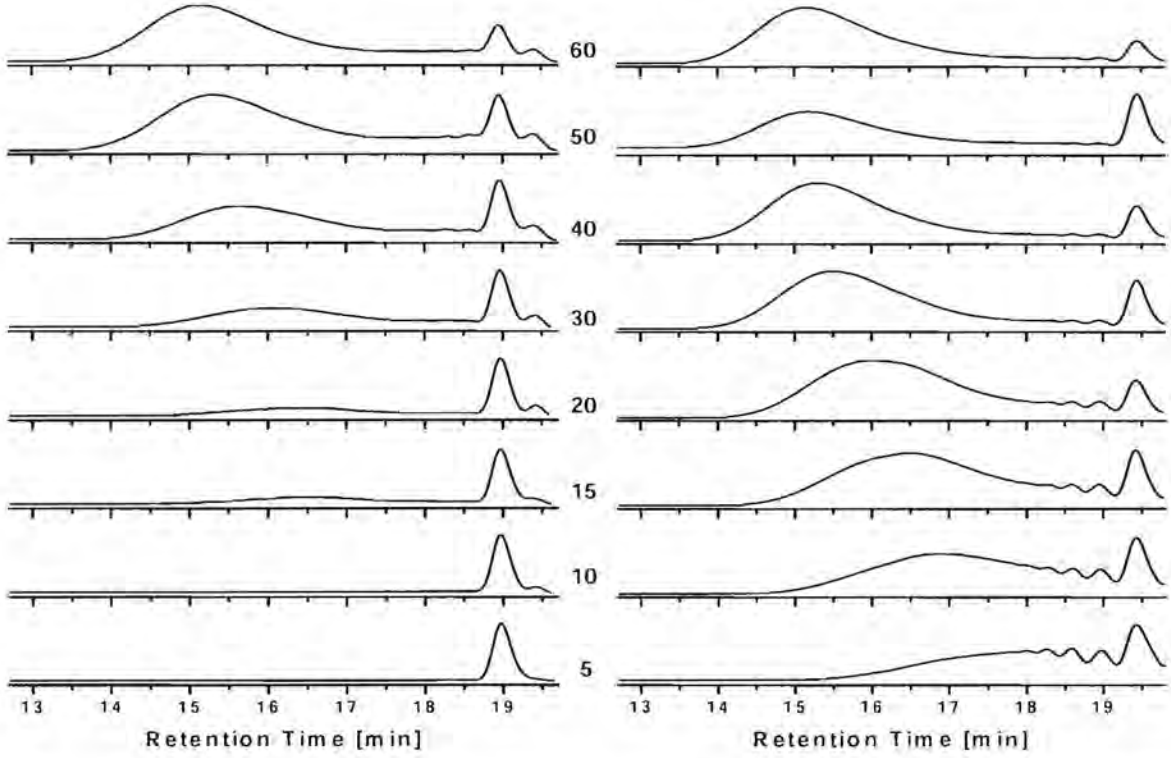


Figure 4-4: *Evolution of molecular weight distribution as a function of reaction time for a typical e-ROP under treated conditions from: left: Initiator **1** (RT(ini **1**) = 19 mins); right: Initiator **2** (RT(ini **2**) = 19.5 mins); determined by SEC in THF.*

4.2.2. Two-pot synthesis of block copolymer P(CL-*b*-MMA)

Poly(ε-caprolactone) macro-initiator with a number average molecular weight of 13.7 kg/mol and a polydispersity index (PDI) of 1.9 was obtained after two hours polymerization and precipitation of the polymer from cold methanol. ^{1}H-NMR analysis of the polymer, after conversion of all end-groups with oxalyl chloride, confirms a low concentration of carboxylic acid end-capped polymers (<2%), implying a high degree of incorporation of initiator **2** into the polymer. The PCL macro-initiator was subsequently used for ATRP of MMA using CuBr/dNbpy as catalyst. An increase in the number average molecular weight from 13.7 to 36.9 kg/mol was observed by SEC (as depicted in Figure 4-5). From ^{1}H-NMR spectroscopy, a

ratio of the block length of PMMA and PCL of 4:1 was determined by comparison of the integrated peak area of the methoxy-protons of the MMA units (δ = 3.62 ppm) with the integrated peak area of the methylene ester protons in the PCL-backbone. This is in good agreement with the monomer feed ratio. In order to directly analyze the PMMA-block of the P(CL-*b*-MMA), the PCL-block was hydrolyzed according to a literature procedure.[17] Comparison of the SEC-chromatogram of the remaining PMMA-block with that of P(CL-*b*-MMA) reveals a shift to lower molecular weight, (24.4 kg/mol), due to the removal of the PCL-block. Although these chromatograms suggest the formation of the block copolymer, the overlapping traces do not provide conclusive evidence for this, exclusively based on the SEC results. Additionally, the combination of SEC and ^{1}H-NMR together hints towards the actual formation of a block copolymer, but no direct proof can be derived from these combined techniques either.

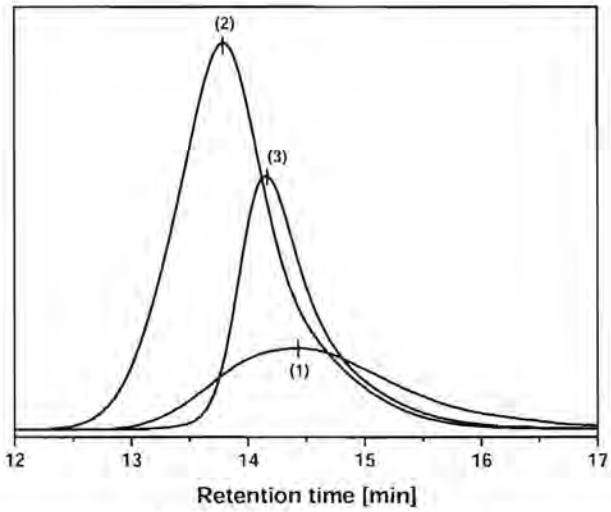


Figure 4-5: *SEC-chromatograms of PCL macro-initiator (1), the block copolymer, P(CL-b-MMA) (2), the hydrolyzed block copolymer, PMMA (3).*

In order to provide additional and stronger evidence for the block structure of the isolated polymers, the polymer was also investigated by gradient polymer elution chromatography (GPEC)[18], which is an interaction chromatography technique that can provide detailed information on the polymer composition. The separation in GPEC is based on both the interaction between the polymer and the stationary phase (adsorption chromatography) and on the solubility of the copolymer in the eluent mixture. Within certain molecular weight limits, it allows the separation of block and homopolymers solely based on their chemical nature. In our case THF/heptane was chosen as solvent/non-solvent system.

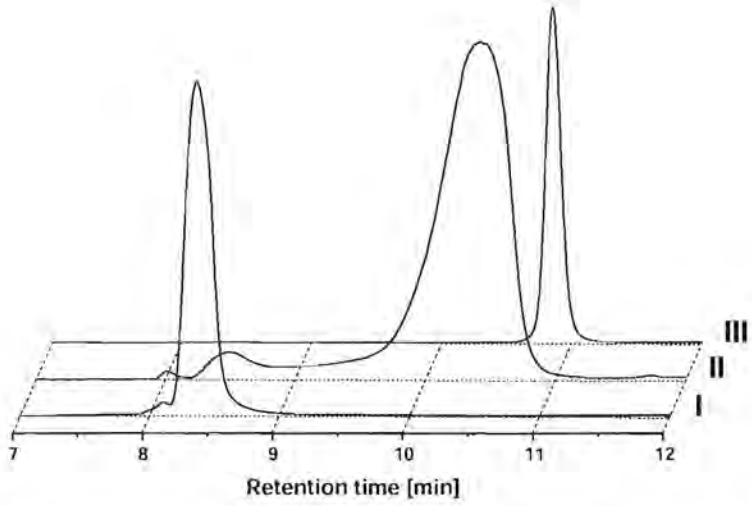


Figure 4-6: *GPEC-chromatograms of homopolymer PCL (I; M_n = 13.7 kg/mol), P(CL-block-MMA) obtained from a two-pot synthesis (II; Mn: 36.9 kg/mol) and homopolymer PMMA (III; M_n = 15.0 kg/mol).*

It was found that, by applying a gradient from 100% heptane to 100% THF over 10 mins, a proper separation of a blend of the two homopolymers could be achieved. Pure PCL (M_n = 13.7 kg/mol) elutes at a retention time of 8.3 mins, pure PMMA (15.0 kg/mol) elutes at 10.8 mins (Figure 4-6). The GPEC-chromatogram of the block copolymer, centered at a retention time of 10.4 mins, is clearly separated from the chromatograms of the two homopolymers. Further inspection of the chromatogram (II) reveals that only small amounts of PCL homopolymer are present in the isolated block copolymer product, which most probably originates from the water-initiated and cyclic PCL.

4.3. Compatibility of lipase

As demonstrated in Scheme 4-1, two synthetic procedures were followed for a one-pot approach (**II**), i.e. consecutive and simultaneous activation of the combined polymerization techniques. In the latter case, all polymerization components will be added to the reaction flask prior to the reaction. In a typical consecutive approach both polymerizations were conducted successively in the same reaction flask, starting with e-ROP followed by ATRP. The controlled radical polymerization was activated by addition of the ATRP-catalyst (route B). In order to warrant high block copolymer yield, the extent of side reactions has to be kept to a minimum. One potential source of side-reactions is the existence of mutual interactions of the reaction compounds. In order to enable a one-pot reaction, initially the compatibility of the enzyme with the reaction components of ATRP and vice versa was investigated under controlled conditions.

4.3.1. Influence of the ATRP-catalyst on e-ROP

A variety of catalyst systems comprising a transition metal salt and an organic ligand can be applied for ATRP[19]. The question is whether and to what extent do they influence the enzyme activity. Hence, a systematic study on the influence of selected ATRP-catalyst systems on the kinetics of the e-ROP of ε-CL was conducted in the presence of a vinyl monomer. As can be derived from Figure 4-7A, a typical enzymatic polymerization of ε-CL at 60 °C in the presence of MMA initiated by initiator **2** achieved almost complete monomer conversion after two hours. When CuBr, a common ATRP-catalyst, was added the reaction stopped at a ε-CL conversion of ca. 20%, after which total enzyme inhibition was observed. Interestingly, an instantaneous enzyme inhibition was observed with $CuBr_2$. This latter observation suggests that the actual inhibiting species is Cu^{2+}, which in the case of the Cu^{+} addition might slowly be generated by oxidation. It also would explain the induction period before inhibition. The exact mechanism of the inhibition is not clear but one could speculate that complexation of the metal cation by the histidine-moiety of the amino acid triad (Ser-His-Asp) in the active site plays a significant role.

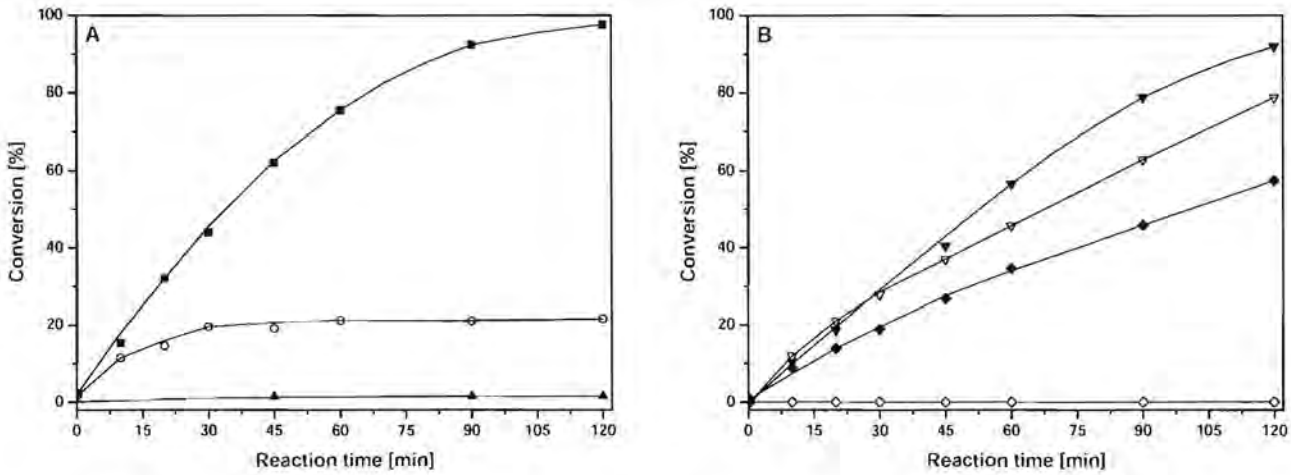
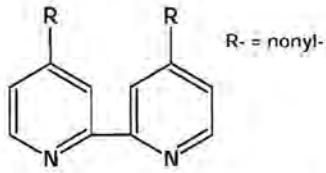


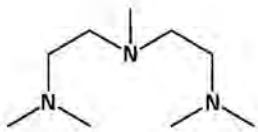
Figure 4-7: *Influence of ATRP-catalyst system on the conversion of ε-CL in the e-ROP in the presence of MMA at 60 °C using initiator 2; A: (■) without ATRP-catalyst; (○) CuBr (1:1-ratio to initiator); (▲) $CuBr_2$ (1:1-ratio to initiator); B: (▼) CuBr/PMDETA (1:1:1-ratio to initiator); (▽) CuBr/dNbpy (1:2.1:1-ratio to initiator); (♦) CuBr/PPMI (1:2.1:1-ratio to initiator); (◊) $NiBr_2(PPh_3)_2$ (1:1-ratio to initiator); ε-CL conversion was determined with ^{1}H-NMR spectroscopy. Lines are drawn to guide the reader's eye.*

In the presence of typical ATRP multivalent ligands like PMDETA, dNbpy, or PPMI, coordinated to copper(I)bromide, enzyme inhibition is much less pronounced (Figure 4-7B). This is not unexpected, as the concentration of metal ions available for inhibition of the enzyme is reduced due to competitive complexation by the ligand. Interestingly, the reaction in the presence of the tridentate PMDETA shows a faster ε-CL conversion than in the presence of dNbpy and PPMI, which is probably due to stronger coordination of the CuBr by

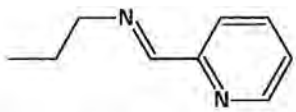
the PMDETA. Finally, the use of $NiBr_2(PPh_3)_2$ as ATRP-catalyst reveals complete inhibition of the enzyme and no monomer conversion after 120 mins reaction. It is known that Ni^{2+} is very strongly coordinated by histidine.[20] Coordination by two trisphenylphosphine moieties is apparently less strong, as the enzyme has lost all of its activity. In conclusion, all ATRP-systems based on coordinated CuBr allow enzymatic polymerization in the tested systems. Since the dNbpy system is known to catalyze a slower ATRP reaction, thus offering more control to the system, this ligand was chosen for the one-pot synthesis of the block copolymer, thereby allowing minor enzyme deactivation during reaction.



4,4'-dinonyl-2,2'-bipyridine (dNbpy)



N,N,N',N',N''-pentamethyldiethylenetriamine (PMDETA)



N-(n-propyl)-2-pyridyl-methanimine (PPMI)

Figure 4-8: *Applied complexation agents for CuBr in ATRP of MMA.*

4.3.2. Influence of the ATRP-monomer on e-ROP

The initial reaction system for the one-pot copolymerization consisted of Novozym 435, ε-CL, initiator **2**, and MMA and, in the case of a simultaneous reaction, ATRP catalyst. While no influence of the ε-CL on the ATRP of MMA was observed, it was not clear whether a simultaneous reaction could be carried out in the presence of MMA without any side-reactions. Hence, a reference reaction was conducted containing all components except the ATRP-catalyst. The obtained product was investigated by MALDI-ToF-MS (Figure 4-9) and compared to a spectrum obtained from a reaction without MMA, but in the presence of *t*-butyl methacrylate (for a reason to be discussed later) (Figure 4-10). For the latter product two main series of peaks can be detected and assigned to the desired polymer, end-capped with the initiator (D) and cyclic polymers (F). Additionally, a minor series of peaks, caused by linear carboxylic acid end-capped polymer, was present (G) (due to water-initiation). In the presence of MMA these water-initiation peaks were hardly detected. Instead, a number of new peaks appeared in the spectrum.

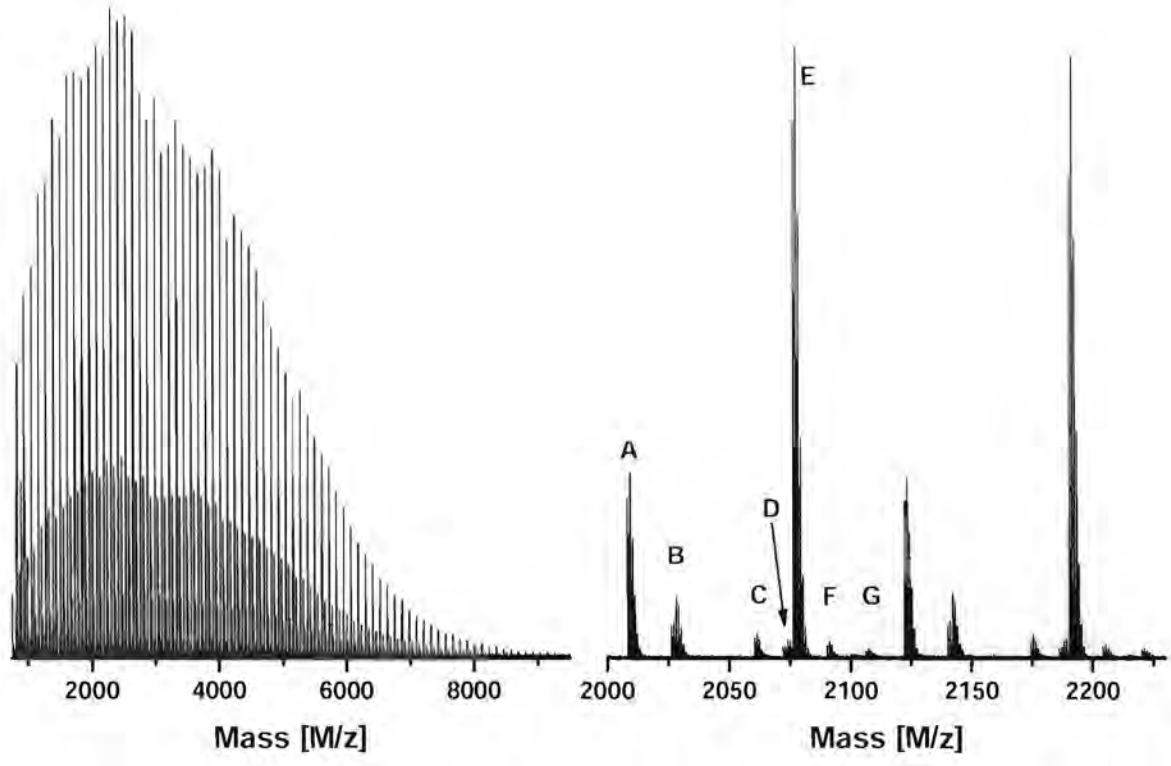
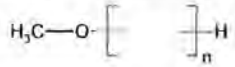
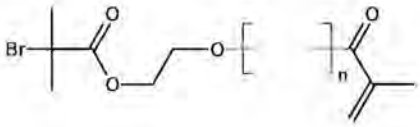
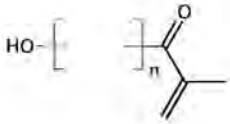
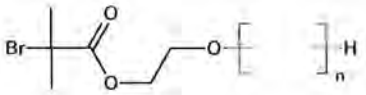
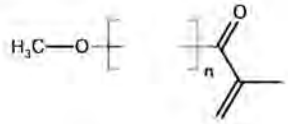


Figure 4-9: *MALDI-ToF-MS spectra of PCL synthesized in presence of MMA using initiator 2 without ATRP-catalyst.*

Careful analysis of this spectrum revealed that the main peak series can be assigned to PCL molecules end-capped with a methacrylate group (B, C, and E). Furthermore, all possible combinations of end-groups can be identified in the spectrum in various intensities. The presence of MMA inevitably results in transesterification, and thus in the incorporation of methacrylate groups to a high extent. Moreover, even the methanol released in this transesterification process acts as an active component in the reaction, as evident from the presence of PCL molecules with methyl ester end-groups (A and E). Consequently, MMA cannot be used for a one-pot cascade reaction. Therefore, the choice of the ATRP-monomer was switched to *tert*-butylmethacrylate (*t*-BMA) as vinyl component, because of the inertness of tertiary alcohol/esters in the enzymatic reaction. Side-reactions similar to MMA have not been observed in MALDI-ToF-MS with this monomer (Figure 4-10).

Table 4-1: *PCL end-groups determined using MALDI-ToF-MS corresponding to the spectrum shown in Figure 4-9.*

Peak	Mass [M/z]	n	End-groups
A	2010	17	
B	2028	15	
C	2064	17	
D	2074	16	
E	2078	17	
F	2092	18	
G	2110	18	

4.4. *Chemoenzymatic block copolymer synthesis in one pot*

4.4.1. Consecutive approach

Taking the results from the control experiments into account, chemoenzymatic one-pot cascade polymerizations were conducted following the two approaches. For the consecutive process ε-CL, *t*-BMA, Novozym 435 and initiator **2** were heated to 60 °C to initiate the e-ROP and obtain the PCL block end-capped with initiator **2**. After 120 mins, CuBr and dNbpy were added in order to activate the ATRP and by that the block copolymer formation.

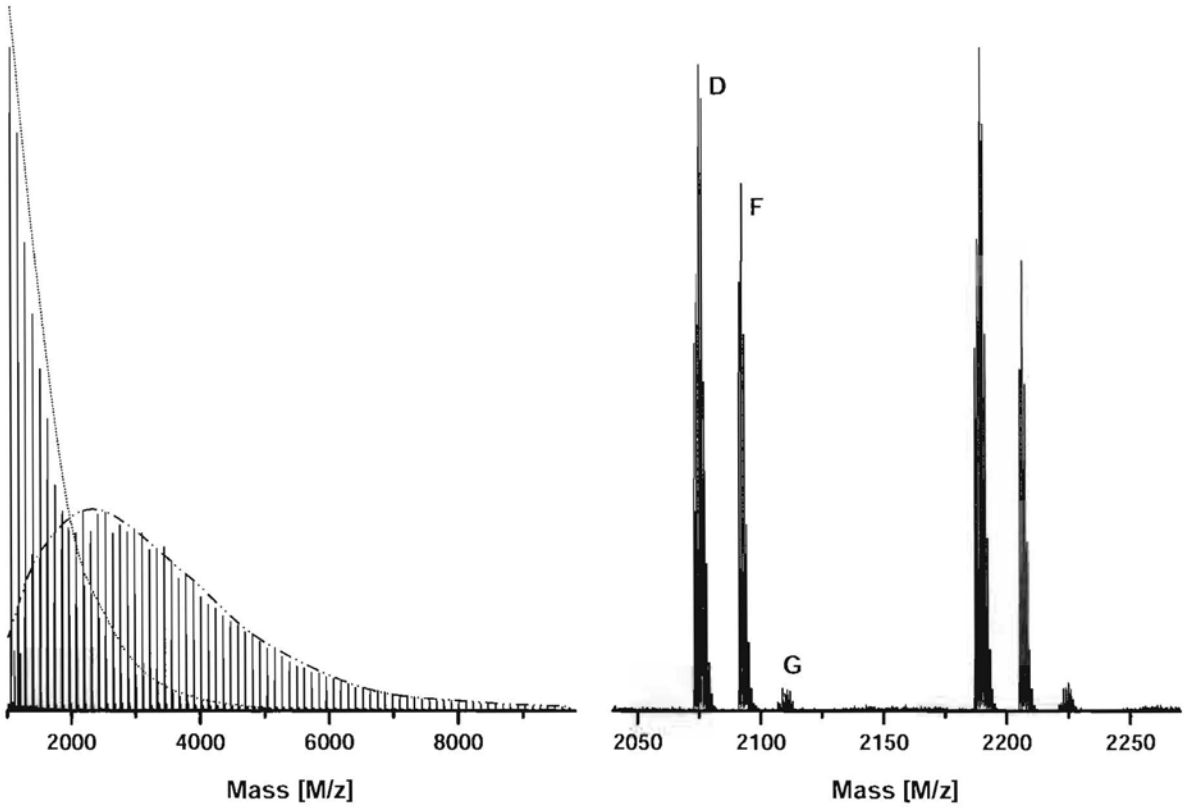


Figure 4-10: *MALDI-ToF-MS spectra of PCL synthesized in presence of t-BMA using initiator 2 without ATRP-catalyst. Lines are drawn to distinguish the different distributions.*

The conversion of both monomers was monitored by ^{1}H-NMR spectroscopy using samples withdrawn from the reaction mixture at predetermined time intervals. To assess the influence of the reaction conditions on the monomer conversion, data were compared with those for a homopolymerization of ε-CL and *t*-BMA. Inspection of Figure 4-11 shows that during the first step of the consecutive process, i.e. the e-ROP, ε-CL conversion reached 95% while only negligible conversion of *t*-BMA was detected. Only upon addition of the ATRP-catalyst, the radical polymerization was started, reaching ~43% conversion within 180 mins (300 mins total). Both reaction rates are comparable to the rates observed from the homo-polymerization under similar conditions, suggesting that both reactions run undisturbed by each other. A clear increase of the molecular weight from 4 200 g/mol (first step) to 6 800 g/mol after the second step was observed in SEC, suggesting the formation of block copolymers.

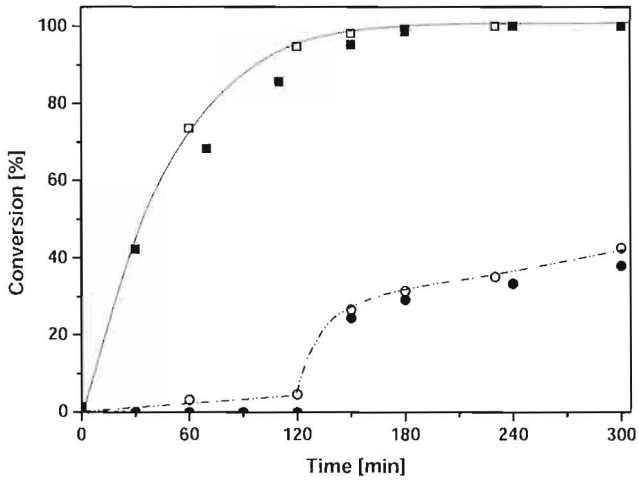


Figure 4-11: *Conversion of ε-CL (□) and t-BMA (○) in a consecutive one-pot polymerization using initiator 2 in comparison with the conversion of ε-CL (■) and t-BMA (●) in a homopolymerization from the same initiator. Lines are given to guide the eye for data set of the cascade polymerization.*

More convincing evidence for the block structure was obtained from GPEC, which allows the separation of polymer based on chemical composition. Figure 4-12 shows the GPEC chromatograms of a blend of PCL (M_n = 13.7 kg/mol) and P(*t*-BMA) (6.7 kg/mol) (chromatogram **II**). Both polymers are separated with a difference in retention time of ca. 2.5 mins, allowing proper separation between the block copolymer and its homopolymers. P(*t*-BMA) elutes after ~11 mins and PCL elutes after ~13.5 mins. Assuming no molecular weight effects, block copolymers comprising PCL and P(*t*-BMA) are expected to elute between the traces of the individual homopolymers. This pattern is exactly observed for the polymer sample obtained from the consecutive approach (**I**). From this result it can be assumed that no significant amounts of neither of the two homopolymers are present and that the block copolymer is rich in PCL, which is also confirmed by ^{1}H-NMR.

4.4.2. Simultaneous approach

When both polymerizations were conducted simultaneously using initiator **2**, i.e. with all components present from the start of the reaction, the situation was different. In this case, it was observed that the ε-CL conversion rate was lower in the simultaneous one-pot reaction compared to the homopolymerization while the *t*-BMA conversion was higher (Figure 4-13). A possible explanation for this behaviour may be the presence of ε-CL, which is reported to work as a cosolvent that promotes the ATRP-reaction.[21] Moreover, comparison of the conversion plots shows that in this reaction the ε-CL conversion stops at 30% after 60 mins reaction time, suggesting enzyme deactivation, while the conversion of *t*-BMA continued. This inhibition of the enzymatic polymerization is most likely caused by steric effects of the P(*t*-BMA)-block, either by physically blocking the enzyme or by inaccessibility of the

initiator end-group for e-ROP. In order to obtain further evidence for the causes of the enzyme deactivation, we performed an e-ROP with P(*t*-BMA) macro-initiator end-capped with initiator **2**. Even after two hours no conversion of ε-CL was observed, indicating that the P(*t*-BMA)-block has an influence on the e-ROP. Obviously, P(*t*-BMA) did not enter the active site of the enzyme. The GPEC results show bimodality (Figure 4-12 - chromatogram **III**): besides the desired block copolymer another trace originating from P(*t*-BMA) homopolymer was formed.

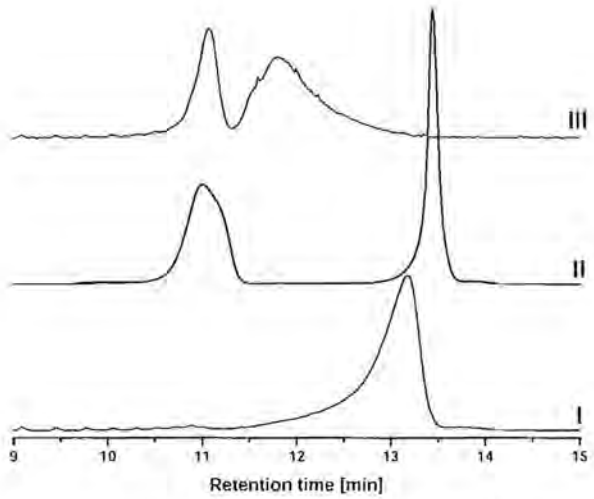


Figure 4-12: *GPEC-chromatograms of polymer obtained from the consecutive approach (I); a blend of homopolymers PCL (M_n = 13.7 kg/mol) and P(t-BMA) (M_n = 6.7 kg/mol) (II); polymer obtained from the simultaneous approach (III).*

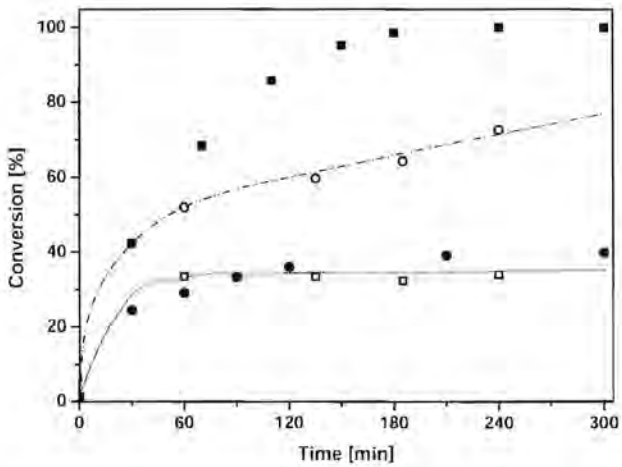


Figure 4-13: *Conversion of ε-CL (□) and t-BMA (○) in a simultaneous one-pot polymerization using initiator 2 in comparison with the conversion of ε-CL (■) and t-BMA (●) in a homo-polymerization using the same initiator. Lines given to guide the eye for data set of the simultaneous cascade polymerization.*

4.5. Discussion and conclusions

The synthetic parameters for the chemoenzymatic cascade synthesis of block copolymers combining enzymatic ring-opening polymerization (e-ROP) and atom transfer radical polymerization (ATRP) in two pots and in one pot were investigated. A detailed analysis of the reaction conditions revealed that a high block copolymer yield can be realized only under optimized reaction conditions. Side reactions, such as the formation of PCL homopolymer, in the enzymatic polymerization of ε-CL could be suppressed to a level lower than 5% by an optimized enzyme drying procedure. Moreover, the structure of the bifunctional initiator was found to play a key role in the initiation behaviour and hence, the purity of PCL macro-initiator. Block copolymers were obtained in a subsequent ATRP-reaction. Detailed analysis of the obtained polymer confirmed the presence of predominantly block copolymer structures.

The design of one-pot chemoenzymatic cascade block copolymer synthesis is possible by a careful choice of the reaction conditions. For the investigated case of e-ROP and ATRP, the influence of the ATRP-catalyst system on the enzyme activity was studied. Possible interference can be minimized by choosing a catalyst system in which copper ions are strongly coordinated (e.g. PMDETA or dNbpy in combination with CuBr). Additionally, interference of the ATRP-monomer can be expected when applying monomers containing ester-moieties (e.g. (meth-)acrylates), which can be activated by the lipase. Steric hindrance by a *tert*-butyl group can avoid this. Nevertheless, this principle can also be advantageous, as it allows the synthesis of novel (meth-)acrylates and (meth-)acrylamides that are difficult to access via chemical techniques. This is be a new field of biocatalytic monomer synthesis that should further be explored.

Block copolymers were obtained in both consecutive and simultaneous approaches, however, with homopolymer impurities in the latter case. The described example also clearly reveals the limitations of the applicability of the one-pot approach. Given the fact, however, that cascade reactions are from the energy and raw material point of view beneficial, reaction design towards cascade reactions remains an important field of research.

4.6. Experimental section

Materials

All chemicals were purchased from Aldrich, stored over molecular sieves and used without further purification unless otherwise noted. Toluene was dried over alumina and stored over molecular sieves (3 Å). Novozym 435 was obtained from Novozymes A/S and was dried as described previously in Chapter 2. Molecular sieves (3 Å) were dried in an oven at 420 °C prior to use. MMA and *tert*-butyl methacrylate (*t*-BMA) were filtered through neutral

aluminum oxide to remove the inhibitor and stored over molecular sieves at 4 °C. Synthesis of bifunctional initiators **1-3** were performed according to literature procedures.[8, 14]

Methods

The water concentrations of the reaction media were determined by Karl-Fischer Coulometry using a Mettler Toledo DL32 Coulometer and Apura CombiCoulomat fritless (Merck) as electrolyte. Size exclusion chromatography (SEC) was performed on a Waters Model 510 pump and Waters 712 WISP, using PL-gel mix D columns (300x7.5 mm, Polymer Laboratories) at 40 °C. THF was used as eluent with a flow rate of 1.0 mL/min. All samples were diluted to 1.0 mg/mL and filtered using 0.2 μm syringe filters. The molecular weights of all polymers were calculated based on polystyrene standards. ^{1}H-NMR spectroscopy was performed using a VARIAN 400 MHz NMR. Data were acquired using VNMR software. MALDI-ToF-MS analysis was carried out on a Voyager DE-STR from Applied Biosystems using trans-2-[3-(4-tert-butylphenyl)-2-methyl-2-propenylidene]-malononitrile (DCTB) as matrix material and potassium trifluoroacetate as ionization agent. All spectra were recorded in the reflector mode. Samples were prepared using 1 mg/mL of polymer in THF. The ratio of polymer sample to matrix was 1 : 5 (w/w). Gradient Polymer Elution Chromatography (GPEC) was performed on an Agilent 1100 Series HPLC with autosampler and an ELSD 2000 detector. A Zorbax RX-Sil column (150x4.6 mm) was used at 25 °C at an eluent flow rate of 1.0 mL/min. An eluent gradient going from pure hexane to pure THF in a period of 10 mins was used, followed by elution with pure THF (2 mins). Data were acquired with HP Chemstation software.

Block copolymer synthesis – Two-pot approach

A copolymer of P(CL-*b*-MMA) was synthesized in a two-pot approach. PCL obtained from initiator **2** was used as a macro-initiator in a subsequent ATRP. The PCL macro-initiator (0.80 g; M_n = 6 800 g/mol; PDI = 2.0) was weighed into a 10 mL two-necked round bottom flask with a magnetic stirring bar, wherein CuBr (16.7 mg; 0.116 mmol) and dNbpy (101.8 mg; 0.249 mmol) were subsequently added (1:1:2.1 molar ratio). The flask, containing all solid reaction compounds, was then deoxygenated by five consecutive argon/vacuum cycles and kept under continuous argon flow. MMA (2.29 g; 22.9 mmol) was degassed with argon for 15 minutes and added to the flask with a syringe. To dissolve all components, the mixture was stirred for 30 minutes at room temperature after which the reaction was started by placing the flask in an oil bath at 90 °C. At the start and the end of the reaction, samples were taken for ^{1}H-NMR and SEC analysis. The reaction was stopped when the polymerization mixture solidified (after approx. 60 mins). The polymer was dissolved in dichloromethane and the solution passed over aluminum oxide to remove the ATRP-catalyst. The obtained polymer

was then precipitated in cold methanol and analyzed by GPEC and SEC. The yield of block copolymer was typically 1.5 g (50%).

Hydrolysis of the block copolymer

The PCL block was hydrolyzed by dissolving the block copolymer (0.60 g) in a 20 mL-mixture of 1,4-dioxane/HCl (37%) (19:1 vol/vol-%). The mixture was then stirred in a 50 mL round bottom flask with a magnetic stirring bar for 24 hours at 85 °C. After hydrolysis, the solvents were removed under vacuum and the crude product was precipitated from cold methanol. The resulting polymer (yield: 0.40 g, 67%) was analyzed with SEC, GPEC, and ^{1}H-NMR spectroscopy.

Enzymatic polymerization of ε-CL in the presence of ATRP-catalyst and MMA

For all enzymatic polymerizations, the amount of Novozym 435 with respect to monomer was kept constant at 10 w/w-%. Prior to the reaction, 0.25 g of Novozym 435 was weighed into the reaction flask together with activated molecular sieves. Both solid ATRP catalyst components, CuBr (35.7 mg, 0.25 mmol) and 4,4'-dinonyl-2,2'-bipyridine (dNbpy; 212.8 mg, 0.5206 mmol) were added to the flask, which was then placed in an oven for 16 hours at 50 °C under vacuum (procedure **7** – Chapter 2). When a liquid ATRP-catalyst component, such as (N,N,N',N",N")-pentamethyldiethylenetriamine (PMDETA; 43.0 mg; 0.25 mmol) was used, this component was added after enzyme treatment. Subsequently, the flask was heated in an oil bath to 60 °C. The reaction was started by adding a stock solution containing 27.0 mg (0.25 mmol) of benzyl alcohol, 2.52 g (22.08 mmol) of ε-CL and 2.29 g (22.89 mmol) of MMA to the flask. At specified time intervals samples (~0.1 mL) were withdrawn from the reaction mixture with a syringe and analyzed by ^{1}H NMR spectroscopy to determine the monomer conversion.

Block copolymer synthesis - Consecutive one-pot approach

For this approach, the enzyme (10% w/w) was dried in the reaction flask together with molecular sieves as described above. After drying, the flask was connected to an argon-vacuum line and five consecutive argon-vacuum steps were performed. Then, the flask was put into an oil bath at 60 °C. A stock solution (5.34 g) consisting of initiator **2** (0.05 M), ε-CL (2.68 M), *t*-BMA (2.58 M) in toluene, was added to a vial and deoxygenated by bubbling with argon for 20 minutes. The reaction was started by adding the stock solution to the flask through a septum with a syringe. At specified time intervals samples (~0.1 mL) were withdrawn from the reaction mixture with a syringe and analyzed by ^{1}H NMR spectroscopy to determine the monomer conversion and by SEC to determine the molecular weight distribution. After 120 mins, a solution of CuBr (0.29 mmol) and dNbpy (0.62 mmol) in toluene (~2 mL) was added to the flask to start the ATRP. After 300 mins (in total), the

reaction was stopped by adding dichloromethane to the reaction mixture and the enzyme was removed by filtration. The solution was passed over aluminum oxide to remove all metal catalysts yielding 2.0 g (79%) of polymer after solvent evaporation. The obtained polymer was then precipitated in cold methanol and analyzed by GPEC, SEC and DSC.

Block copolymer synthesis - Simultaneous one-pot approach

For this approach, 169 mg enzyme was dried in the reaction flask together with molecular sieves, 42.7 mg CuBr (0.30 mmol) and 254.5 mg dNbpy (0.62 mmol) as described above. After drying, the flask was connected to an argon-vacuum line and five consecutive argon-vacuum steps were performed. A stock solution (5.22 g), consisting of initiator **2** (0.055 M), ε-CL (2.686 M), t-BMA (2.587 M) in toluene, was deoxygenated by bubbling with argon for 20 mins. The reaction was started by adding the stock solution to the flask through the septum with a syringe. At specified time intervals samples (~0.1 mL) were withdrawn from the reaction mixture with a syringe and analyzed by ^{1}H-NMR spectroscopy to determine monomer conversion and by SEC to determine molecular weight distribution. After 120 mins, the reaction was stopped by adding dichloromethane to the reaction mixture and removing the immobilized enzyme by filtration. The solution was passed over aluminum oxide to remove all metal catalyst yielding 1.65 g of polymer (89%). The obtained polymer was then precipitated in cold methanol and analyzed by GPEC, SEC and DSC.

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5

Poly(ω-pentadecalactone) Synthesis and Properties of a High Molecular Weight Aliphatic Polyester

Abstract

Poly(ω-pentadecalactone) (PPDL) has a remarkable structure, as its monomeric unit consists of 14 methylene-groups and one ester-moiety. Hence, a linear poly(ethylene)-like biopolymer has come available, which has hardly been investigated before. In order to investigate its material properties, high molecular weight polymer is required, which cannot be obtained via chemical ring-opening polymerization. Surprisingly, lipases have shown remarkable activity towards PDL. In this study, we push the molecular weight of PPDL to new limits using enzymatic ring-opening polymerization (e-ROP). Moreover, parameters that influence these limits are investigated and optimized. On a small scale, a product with a weight average molecular weight (M_w) of 276 kg/mol (PDI = 38) was isolated. The molecular weight of this product was the highest observed in this study. The polymerization is scaled up to obtain enough material for material testing. Diffusion limitation is reduced and PPDL with M_w = 190 kg/mol (PDI = 3.2) is obtained with yields of almost 30 g. In addition, the thermal and mechanical properties, as well as the hydrolytic degradation behavior of PPDL have been investigated. Evaluation was performed by comparing all available data of PPDL, to the data of other aliphatic polyesters (PLA and PCL), and poly(ethylene) in order to determine specific applications for this novel material.

5.1. Introduction

One of the emerging new areas is the application of polymeric materials for medical purposes.[1] Since fifty years biodegradable polymers for medical applications have attracted much attention.[2] Drivers for this trend include the need for the development of biodegradable materials that are biocompatible for application in the human body without losing their short term properties. Another challenge in the application of biodegradable polymers in medical applications is the concern about the toxicity of degradation products and extractables, such as unreacted monomer, anti-oxidants, initiator-fragments, and the catalyst. Typically, this toxicity is more pronounced for the low molecular weight compounds of the polymeric material, as these can interact with the outer cell surface or even enter into the cell interior. Hence, a critical balance must be found when dealing with biodegradable polymer for biomedical applications, in avoiding toxicity of the degradation products on one hand, and realizing biocompatibility on the other. Several biomedical target areas can be considered wherein biodegradable polymers can be applied, such as suturing, orthopedic devices, controlled drug delivery, stents, and tissue engineering.[3] Among the biodegradable polymers that have been applied and are approved for medical use are aliphatic polyesters.

Generally, two routes are applicable for the synthesis of polyesters: (i) polycondensation of hydroxy-acids or diols and diacids and (ii) ring-opening polymerization (ROP). While polycondensation is applied on a large scale for a number of technical polyesters due to the availability of a variety of low-cost monomers, ROP is limited to cyclic esters, providing significant advantages over polycondensation as higher molecular weights can be obtained. Additionally, the production of oligomers is avoided in ROP, whereas in polycondensation a very high end-group conversion is necessary to achieve a respectable molecular weight and to avoid the presence of oligomers in the final product.

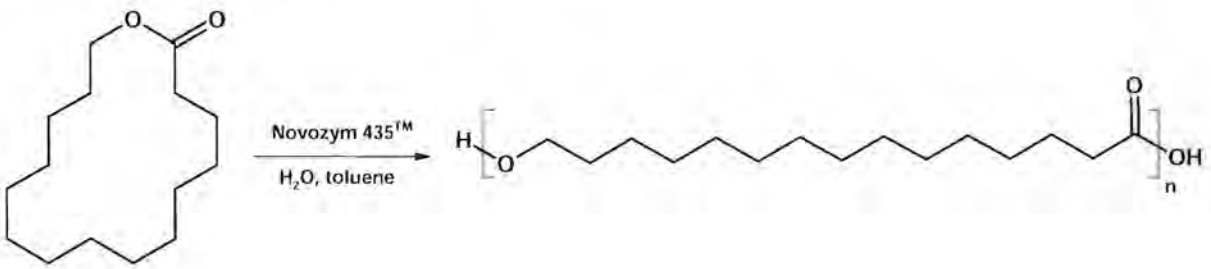

Figure 5-1: *Structure of ω-pentadecalactone (PDL) applied in the enzymatic synthesis of poly(ω-pentadecalactone) (PPDL).*

Figure 5-1 shows the structure of ω-pentadecalactone (PDL) and the corresponding polymer, PPDL. The structural similarity to linear polyethylene (PE) is clearly noticeable. However, while PE is not biodegradable, PPDL comprises ester-moieties which make PPDL

potentially degradable. Several groups have reported on the rate of polymerization of PDL, via chemical as well as via biocatalytic methods.[4-8] It was observed that PDL and other macrolides behave differently from smaller lactones (e.g. δ-valerolactone and ε-caprolactone).[7] Lebedev reported the chemical ring-opening polymerization (c-ROP) using diethyl zinc, although no details about the synthesis nor polymer were mentioned.[9] Duda et al. used zinc octoate/butyl alcohol for c-ROP and reported low activity of the catalyst towards PDL, resulting in low monomer conversion after long reaction times at elevated temperatures (26% after 7 days at 100 °C).[8] In addition, the molecular weight of the obtained PPDL was low (~ 1350 g/mol). Zhong et al. showed that high PDL-conversion and respectable molecular weights (M_n = 30 kg/mol) could be obtained by using yttrium isopropoxide.[6] Good control over the polymerization was reported, allowing chemical synthesis of PPDL. Nevertheless, the use of yttrium without purification as metal-catalyst makes the application of PPDL for biomedical purposes impossible. On the contrary, enzymatic synthesis of PPDL with Novozym 435 was reported to be very fast at mild conditions (>80% PDL-conversion after 20 mins at 55 °C). Molecular weights (M_n) up to 86 kg/mol (PDI = 2.37) were reported by Kumar et al. after precipitation in methanol.[10] Also the effect of immobilization was investigated, revealing that immobilized enzyme (lipase PS) has higher activity towards PDL and gives higher molecular weight polymer than non-immobilized enzyme.[5] Enzymatic polymerization of PDL in miniemulsion with *Pseudomonas Cepacia* lipase was reported, giving PPDL with M_n of 200 kg/mol.[11] Also other enzymes have been utilized as biocatalyst, although activity of these enzymes towards PDL was lower than Novozym 435.[12]

The structure of PPDL suggests similarities with linear polyethylene (PE), as rather long aliphatic moieties (14 methylene-units) are interconnected by ester bonds. It is a crystalline polymer with a good thermostability that melts around 100 °C and has a glass transition temperature far below room temperature (-27 °C).[9, 13, 14] The crystallization behavior of PPDL was compared to that of PCL and PE and revealed large similarities with PE, despite the use of low molecular weight PPDL (M_n = 5500 g/mol).[15] The crystal structure of the polymer was determined using powder wide angle X-ray scattering (WAXS), and showed that chain arrangement is quite similar to that of poly(ε-caprolactone) (PCL) and PE.[16]

Beyond similar properties with respect to PE, PDL can also be employed for the synthesis of copolymers. Examples include copolymers of PDL with ε-caprolactone obtaining random copolymers[10, 17, 18], with trimethylene carbonate (TMC) to produce both random and block copolymers[19, 20], with polybutadiene macro-initiator resulting in block copolymers[21], with methacrylated PEG obtaining graft copolymers[22], and with 2-oxo-12-crown-4-ether to produce random copolymers.[23] These examples prove the applicability of this hydrophobic monomer to obtain polyethylene-like biomaterial.

In this chapter, the enzymatic synthesis of PDL will be carefully re-examined to obtain insight into all steps of the synthesis and the obtained products. First, the polymerization

behavior of PDL and ε-CL will be compared under normal conditions. Subsequently, a small-scale synthetic approach is investigated with the goal to push the molecular weight of PPDL as high as possible (1-2 g of product). The obtained information will be utilized to produce high molecular weight PPDL on a larger scale (30 g product), such that it can be applied to test its material properties, e.g. after fiber spinning. Reported data on thermal and mechanical properties are summarized and combined with the new investigated properties, such as the degradation behavior of PPDL. Finally, all data are evaluated and compared with existing polymer materials such as poly(L-lactic acid), poly(ε-caprolactone) and poly(ethylene) to determine possible applications for high molecular weight PPDL.

5.2. Enzymatic synthesis of PPDL

In order to investigate the enzymatic synthesis of high molecular weight PPDL, first e-ROP of PDL was performed in toluene using benzyl alcohol as nucleophile and Novozym 435 as catalyst at 60 °C. The rate of the PDL-consumption was compared with that of an e-ROP of ε-CL under the same conditions. The enzyme was used without pre-treatment (i.e. water was present in the enzyme) and the reactions were performed on laboratory scale (1-2 g of product). Figure 5-2A shows the monomer consumption as a function of reaction time. As expected, the conversion of PDL is faster than the conversion of ε-CL under the same conditions. After 20 mins of reaction, the viscosity of the PDL-system increased to such an extent that magnetic stirring became impossible. Therefore, PDL-polymerization was stopped after 30 mins, at which time almost full conversion was achieved.

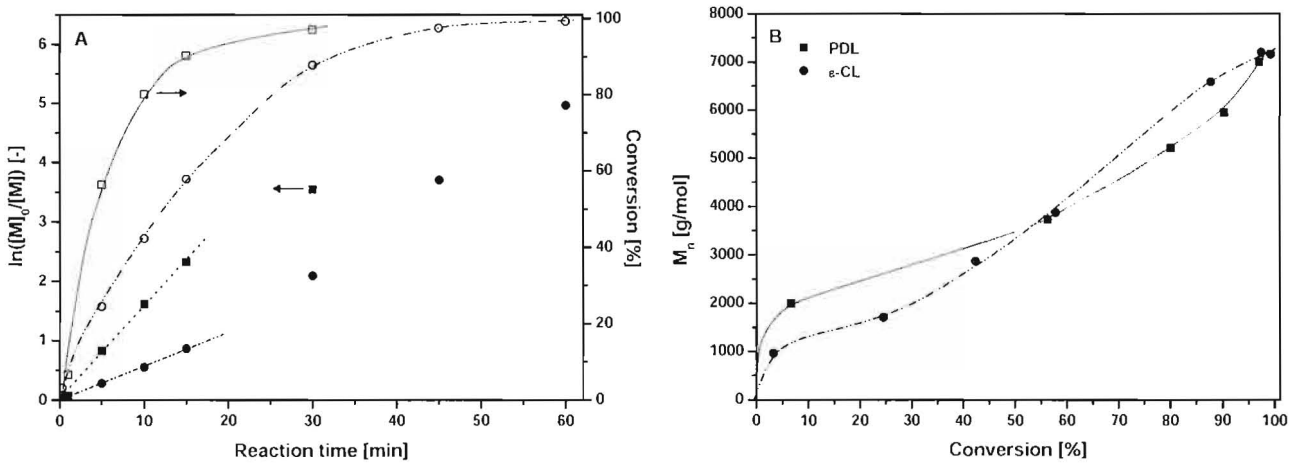

Figure 5-2: *A: Evolution of monomer consumption as a function of reaction time; ■: PDL; ●: ε-CL; open symbols represent conversion, solid symbols represent ln([M]$_0$/[M]); B: Evolution of molecular weight as a function of monomer conversion; Both reactions were performed at 60 °C in toluene with the same target M_n = 10 000 g/mol; Monomer conversion and molecular weight were determined by ^{1}H-NMR; lines are added to guide the eye.*

The target molecular weights were 10000 g/mol for both polymerizations and was set by adjusting the monomer to initiator ratio (M_{PDL} = 240 g/mol; M_{CL} = 114 g/mol). Figure 5-2B demonstrates that the final molecular weight is similar for both end-products. The observed initial increase can be explained by the final conclusion of Chapter 3: initially, water present in the enzyme starts the propagating chain, whereas the added initiator, having lower activity than water, is built in later (by either initiation or transesterification).

Although the obtained polymers have low molecular weights (~7000 g/mol from ^{1}H-NMR spectroscopy), PPDL was very sparingly soluble in many solvents at room temperature. Several solvents were tested to investigate the solubility of PPDL, by adding 1 mL of solvent to an accurately known amount of PPDL (20 mg; M_n ~7000 g/mol). After shaking for 5 days, the liquid phase was removed by filtration over a 0.2 μm filter. Subsequent evaporation of the solvent allowed the determination of the PPDL fraction that was dissolved. Chloroform was observed to be the only solvent that could dissolve PPDL to a reasonable extent (~15 mg/mL) at room temperature (Figure 5-3). The reason for this behavior is expected to be the high crystallinity of the material (~65% from DSC-analysis[13]). The low solubility of PPDL even for low molecular weight has to be taken into account when designing reactors for the synthesis of high molecular weight PPDL and for subsequent molecular characterization. Clearly only at elevated temperatures solubility increases with a sufficient amount, allowing extension of the PPDL-chains.

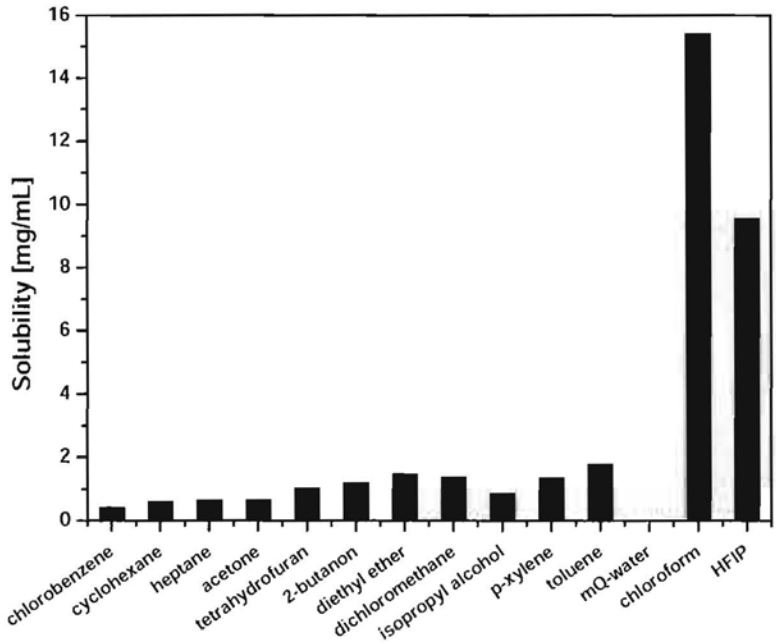

Figure 5-3: *Solubility of PPDL (M_n = 7000 g/mol) in different solvents at room temperature, after 5 days of shaking. For data acquisition see text.*

5.3. *Enzymatic synthesis of high molecular weight PPDL*

The long, linear aliphatic chain of PPDL, which causes the high crystallinity, gives rise to the expectation that PPDL could reveal similar fiber properties as polyethylene (PE). It is commonly known that to obtain good fiber properties, not only high molecular weight polymer is required, but the polymer should also lack short polymer chains which function as plasticizing agent. The approach that can be followed to obtain high molecular weight polymers in e-ROP is similar to the approach that is followed in chain polymerization by using a very high monomer to initiator ratio. The theoretical M_n can be expressed as:

$$M_n(\text{th.}) = \frac{[\text{Mon}]_0}{[\text{Ini}]_0} X_{\text{Mon}} M_{\text{Mon}} + M_{\text{Ini}} \qquad (5.1)$$

where X_{Mon} represents the monomer conversion and M_i is the molecular weight of compound i. In this approach, water is the initiator and by the combination of enzyme pre-treatment and drying of all reactants, the concentration of water can be minimized. The dual role of water in e-ROP was discussed in Chapter 2 and 3, where water was regarded as a competitive nucleophile with respect to other nucleophiles. Now, water is the sole nucleophile, i.e. small amounts of water that cannot be removed without losing enzyme activity will function as nucleophile. Theoretically, this approach will lead to the highest possible molecular weight that can be achieved in this system. Previously, it was shown using TGA that the enzyme can be dried up to a minimum of ~1.2 w/w-% of water based on the total weight of the enzyme and the support. Therefore, by using a very small amount of enzyme and careful drying of all the components in the reaction mixture, the total amount of water can be limited and accurately controlled.

5.3.1 Small-scale experiments

Initially, e-ROP of PDL to high molecular weight was performed on a small scale (1-2 g of product) at 70 °C. As sampling was found to be difficult due to the high crystallinity of the polymer, parallel reactions were performed to obtain insight in the rate of polymerization during the reaction. In Figure 5-4, the evolution of monomer consumption and molecular weight during the reaction are depicted. It is observed that in these polymerizations longer reaction times are needed to achieve complete conversion (Figure 5-4A). Figure 5-4B shows that via this approach, high molecular weight PPDL can be synthesized. TGA revealed that after enzyme treatment ~0.11 w/w-% of the enzyme was lost, as we believe by the evaporation of free water (Chapter 2). This water is expected to take part in the polymerization. In the polymerizations depicted in Figure 5-4, 10 w/w-% of enzyme to monomer was used, which corresponds to a maximum of 0.011 w/w-% of free water to monomer. From this ratio, the expected molecular weight can be calculated using equation

5.1, which corresponds to a molecular weight of at least 164 kg/mol at complete conversion. In Figure 5-4B, it is shown that maximal peak molecular weights (M_p) of 280 kg/mol were achieved (calibrated on PS-standards). This value reflects the most prominent molecular weight in the polymer. The evolution of the M_n needs extra discussion.

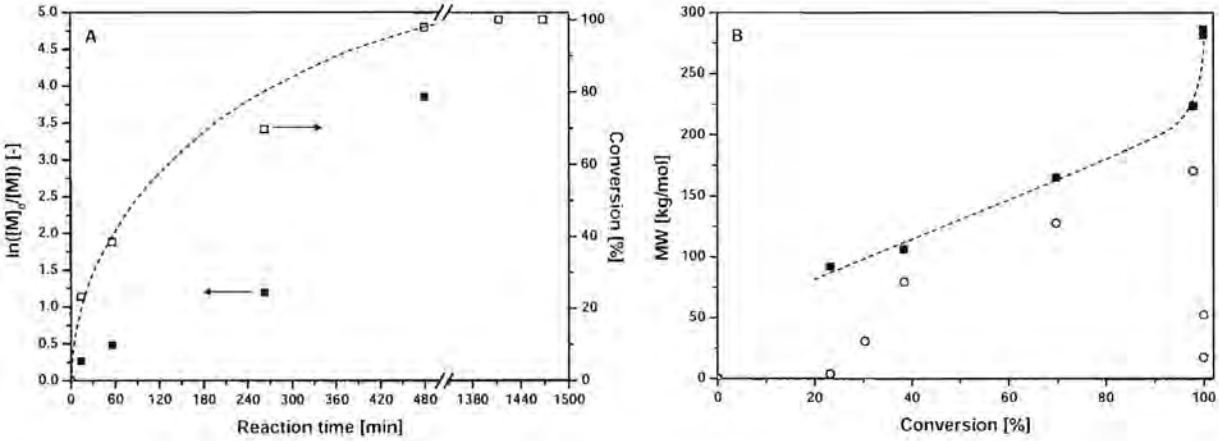

Figure 5-4: *A: Evolution of PDL consumption as a function of reaction time; ■: ln([M]$_0$/[M]; □: monomer conversion; B: Evolution of molecular weight as a function of PDL conversion; ■: M$_p$; ○: M$_n$; All data points were acquired from single reactions at 70 °C in toluene; PDL conversion was determined by ^{1}H-NMR and molecular weights by SEC in chloroform at 25 °C, based on PS-standards; lines are added to guide the eye.*

Unfortunately, it was not possible to determine the M_n of the polymer accurately using ^{1}H-NMR spectroscopy as a result of the large ratio of repeating units to end-groups when reaching high molecular weights. Size exclusion chromatography (SEC) of high molecular weight PPDL samples in chloroform at 25 °C reveals a similar evolution for M_p as was observed for the M_n in an enzymatic polymerization of PDL with benzyl alcohol (Figure 5-2B). After a fast initial increase, the molecular weight increases more or less linearly with increasing monomer conversion. At almost full conversion, the molecular weight of the polymer seems to further increase over time. This increase might be induced by a condensation reaction of hydroxy- and carboxylic acid end-groups, thereby producing water. Hence, the polymerization should be stopped at a maximum of 95 % monomer conversion in order to maintain control over the polymer product.

As reported above, molecular weights were determined by SEC at 25 °C using chloroform as eluent, which is in agreement with almost all literature reports.[6, 10, 11] The analysis showed irreproducible results for M_n, and often broad molecular weight distributions were observed. A closer look at the system revealed a large pressure built up during SEC-analysis of PPDL-samples, which may indicate that the polymer did not dissolve properly in chloroform at 25 °C. The prepared samples of PPDL in chloroform (1 mg/mL) were clear and transparent, but this observation does not exclude the formation of very small swollen particles (not dissolved) and polymer aggregates. When these samples were filtered over a 0.2 μm syringe filter, a

large resistance was observed, which is an additional indication that the polymer did not dissolve well. Only Van der Mee et al. reported on the poor solubility of PPDL[23], after which they decided to use elevated temperature (80 °C) and another eluent (*o*-dichlorobenzene)[7]. Therefore, in the rest of this study, molecular weight analyses were performed using SEC at 160 °C with trichlorobenzene (TCB) as eluent - common for PE - to overcome the crystallinity and consequent insolubility problems. Nevertheless, the molecular weights that were detected in the chloroform-system can be compared with results from literature obtained in the same solvent (Table 5-1). Although these absolute values should be interpreted with great care, it can be concluded that the M_n-values in this investigation are the highest obtained via e-ROP of PDL in solution.

Table 5-1: *Results from synthesis of PPDL: comparison of literature values based on SEC in chloroform at 25 °C and calibration on polystyrene standards; c = chemical ROP, e = enzymatic ROP, m = prepared via miniemulsion.*

Entry	ROP	t_r [hr] [a]	T_r [°C] [b]	M_n [kg/mol] [c]	M_w [kg/mol] [c]	M_w/M_n [-] [b]	Ref.
1	c	168	100	1.4 [d]	2.7 [d]	1.9 [d]	8
2	c	0.5	80	32	51	1.6	6
3	e	2	70	86	204	2.4	10
4	e	1	70	62	118	1.9	5
5	e,m	12	60	200	-	-	11
6	e	4.3	70	51	86	1.7	*Present*
7	e	8	70	170	292	1.7	*study*
6 *	e	4.3	70	2.8 *	50.4	18.0 *	*Present*
7 *	e	8	70	7.2 *	276	38.3 *	*study*

[a] Reaction time in hours; [b] Reaction temperature in °C; [c] SEC in chloroform at 25 °C in calibration on PS-standards; [d] SEC in dichloromethane at 20 °C, and calibration on PCL-standards; * SEC in trichlorobenzene, and calibration on PS-standards; n.d.: no data available.

When SEC-analysis of entries **6** and **7** (data from Figure 5-4) was performed in trichlorobenzene (TCB) at 160 °C, the analysis revealed very low M_n-values (2.8 and 7.2 kg/mol) indicating that a large amount of low molecular weight polymer was present. As a consequence, the polydispersity index was extremely high. The reason for this broad molecular weight distribution can be found in the lack of agitation in the system. After 10-15 mins, the polymerization mixture had become so viscous that the magnetic stirrer was blocked. As a result, diffusion of polymer into and out of the enzyme particles was hindered. Probably, the lack of mobility of the polymer chains in the enzyme particles allowed these chains to continue growing as they were close to the active site of the enzyme, hindering the entrance of the enzyme and possibly even the particle by shorter polymer chains. Hence, polymer chains in the 'solution' were prevented from entering the enzyme particle, and could not continue growing. The data in Figure 5-4A reveal that monomer was continuously

consumed, from which it can be concluded that monomer was still able to diffuse into the enzyme active site, containing 'trapped' polymer

In conclusion, when performing e-ROP of PDL on a small scale, the obtained high molecular weight PPDL had a very high polydispersity index and high M_w. SEC in chloroform at 25 °C on these samples was proven not to be a good option as high molecular weight PPDL appeared not to dissolve (well) at this temperature. SEC at elevated temperatures, e.g. at 160 °C in TCB, analogue to molecular weight analysis of polyethylene, proved to be the better technique.

5.3.2 Up-scaled synthesis

In order to obtain enough polymer product to analyze its material and thermal properties, the enzymatic polymerization was scaled up. To obtain a high molecular weight polymer with a narrower molecular weight distribution (polydispersity ~2.0), mass transport limitations, both in the enzyme particle (internal) as in an outer stagnant film (external), should be avoided. Two approaches can be followed to reduce the mass transfer limitations: (1) decreasing the particle size and (2) achieving good mixing. Intraparticle mass transport limitation depends strongly on the size of the catalyst particle, as the molar flux of a compound in a catalyst particle is inversely correlated with the size of the particle.[24-26] The average particle size of Novozym 435 is ~400 μm (varying from 100 - 1000 μm), but it is reported that the enzyme is located in the outer shell of the particle of ~80 μm.[27] Therefore, it is not possible to reduce the (effective) particle size without grinding the particles. In this study, this has not been performed, as it was believed that this reduces enzyme activity.

As the increasing viscosity with conversion makes the system difficult to mix, a Teflon anchor stirrer (overhead) was used to obtain mixing throughout the entire mixture. This would also avoid grinding of the immobilized enzyme during the polymerization. Moreover, since the obtained PPDL was difficult to dissolve, a relatively large reactor volume (250-300 mL) was used for the reaction. This allowed the addition of extra solvent after the reaction, so that the polymer and enzyme could subsequently be separated by filtration. In addition, a double-wall glass reactor vessel was chosen such that a thermostatic bath with oil could heat up the reactor, as this enables a more stable heat transfer into the system (also after adding additional solvent after the reaction). Initially, the reaction temperature was kept at 70 °C. An enzyme to monomer ratio of 1 : 20 (w/w-%) and an equal monomer to solvent (toluene) weight ratio was used. The total reaction volume was around 70 mL. In Table 5-2, the results of e-ROP of PDL to high molecular weight PPDL are listed for different reaction conditions (entries **U1-U5**). In all polymerizations complete monomer conversion was obtained after ~70 hours (3 days) of reaction (determined by ^{1}H-NMR spectroscopy).

Table 5-2: *Conditions and results from up-scaled e-ROP of PDL.*

Entry	T_R [°C]	M_n[1] (kg/mol)	M_n[2] (kg/mol)	M_w[2] (kg/mol)	*PDI* [-][2]	Remarks
U1	70	16.5	2.4	21.0	8.8	*Stirrer too low → enzyme grinded*
U2	70	29.8	3.3	45.7	13.8	*Reaction stopped with para-oxon*
U3	70	48.8	15.3	56.4	3.7	*Polymer precipitated in methanol*
U4	80	87.8	19.6	85.7	4.4	*Molecular sieves were added to enzyme*
U5	85	81.1	31.3	91.2	2.9	*No crystallization during reaction*
6[3]	70	90.4	5.4	136.3	25.2	*Laboratory-scale experiment*
6[4]	70	90.4	4.9	270.7	55.2	*as reference for MWD*

[1] Determined by ^{1}H-NMR at 20 °C in $CDCl_3$; [2] Determined by HT-GPC in TCB at 160 °C, calibrated on polyethylene standards; [3] Polymerization on laboratory-scale; [4] Same entry **6**, but SEC in TCB at 160 °C calibrated on polystyrene standards.

In entry **U1**, the clearance between the stirrer and the bottom of the reactor was too small, which caused grinding of the enzyme during the reaction. After 72 hours, no original enzyme particles were observed in the obtained slurry. Filtration of the obtained slurry-solution was difficult as a result of plugging of the filter by grinded enzyme particles. The molecular weight of the obtained polymer was 16.5 kg/mol (^{1}H-NMR spectroscopy). In entry **U2**, the reaction was stopped by the addition of para-oxon, an enzyme-inhibitor that reacts irreversibly with the active site, followed by filtration of the enzyme and subsequent neutralization of para-oxon with methanol. The observed molecular weight was higher, i.e. M_n = 29.8 kg/mol. In entry **U3**, the polymerization was terminated similarly as in entry **U2**, but the obtained polymer was precipitated using the methanol-phase (that was added for neutralization of para-oxon). Hence, the lower polydispersity index reduced from 13.8 to 3.7. Nevertheless, in all entries (**U1-U3**), the polymer crystallized on the stirrer shaft, as the reaction temperature was below the crystallization temperature of PPDL ($T_r < T_{cr}$). In order to prevent crystallization during the reaction, the reaction temperature was increased to 80 °C (entry **U4**), but crystallization was still observed on the stirrer shaft. Additionally, molecular sieves were added to this system, in order to have even less water participating in the reaction. A sharp increase in molecular weight was observed when using this procedure (M_n = 87.8 kg/mol). However, at the end of the reaction the stirrer was found to be broken into two parts and a fraction of the molecular sieves and enzyme particles were grinded (either by the increased shear stress or by the demolished stirrer blade). Filtering this slurry was difficult and was not complete, resulting in small grinds in the final polymer (resulting in a brownish color). In addition, the polymer was very difficult to dissolve in p-xylene at 80 °C. Nevertheless, the molecular weight of the polymer increased, probably due to the addition of the molecular sieves. In entry **U5**, only 1 w/w-% of Novozym 435 was used with respect to monomer, and molecular sieves were added to the reaction to further reduce the amount of water in the

system. Moreover, the reaction temperature was further increased to 85 °C and crystallization was avoided. The molecular weight of the obtained polymer was 81.1 kg/mol and the precipitated polymer was white, although monomer residues could still be smelled. Surprisingly, monomer was not determined with ^{1}H-NMR spectroscopy.

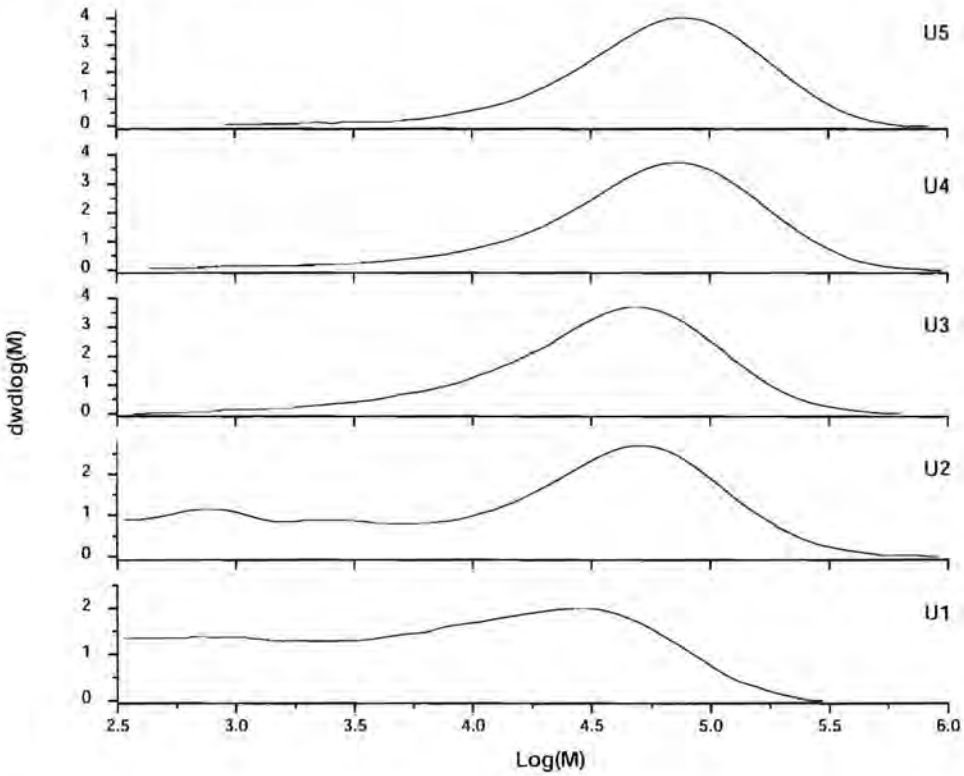

Figure 5-5: *Molecular weight distributions of different up-scaled enzymatic polymerizations of PDL; determined by HT-GPC in TCB at 160 °C, polymers were calibrated on PE-samples ; Entry numbers are given in the top right corner of each distribution and correspond to entries in Table 5-2.*

Polymer samples were sent out to determine the molecular weight distribution using high-throughput SEC (HT-SEC) in TCB at 160 °C (former SEC-setup in TCB was not available). All polymers were calibrated to polyethylene standards, which complicated comparison to previous results. Fortunately, a relationship is known for the correlation of the molecular weight of polystyrene and polyethylene for SEC in TCB at 160 °C. This relationship is expressed by[28]:

$$\log(M_{PE}) = 0.1760 + 0.9064 \times \log(M_{PS}) \qquad (5.2)$$

where M_{PE} represents the molecular weight of polyethylene and M_{PS} represents the molecular weight of polystyrene. A reference sample was added, for which the molecular weight based on PS-calibration was known (entry **6**). The molecular weight of the polymer in entry **6** determined based on PS-standards and PE-standards corresponds with the correlation

expressed in equation 5.2. Nevertheless, it is not known how this exactly relates to the real value of the molecular weight of PPDL. An overall trend is visible with respect to the molecular weight distribution. In Figure 5-5, the molecular weight distributions of the products of entries **U1-U5** are depicted. A clear shift in molecular weight can be observed. Also, the difference in polydispersity index is evident from these molecular weight distributions.

When converting the molecular weights from PE- to PS-calibration using equation 5.2, it was found that the highest molecular weight M_n was 58.3 kg/mol (entry **U5**). The corresponding M_w was 190 kg/mol, so the polydispersity index was 3.2. It can therefore be concluded that high molecular weight PPDL was synthesized in an almost 30 grams yield, which can be spun into a fiber as low molecular weight fraction are barely present. This polymer was used to determine the thermal and mechanical properties of PPDL, see the next section.

5.4. Properties of high molecular weight PPDL

Because of its structural similarities towards polyethylene (PE), many material properties of PPDL have been investigated and reported in literature. In this section, all available data on the properties of PPDL are summarized and combined in order to find possible applications for this novel polyester (section 5-5).

Table 5-3: *Overview of thermal properties reported in literature and based on own data discussed in this evaluation.*

Entry	M_w [kg/mol]	T_m [b] [K]	T_g [K]	T_{degr} [d] [K]	ΔH_m [b] [J/g]	χ_c [e] [-]
Lebedev et al. [9]	$[\eta] = 0.93$ [a]	370.5 *	251 [b]	-	175	75
Skoglund et al. [15]	$[\eta] = 0.20$ [a]	359	250 [b]	-	153	65.5
Zhong et al. [6]	30 #	371	n.d. [b]	-	178	77
Focarete et al. [13]	129 #	370	246 [c]	675-725	149	64
this study	190 *	368	248 [c]	660-725	138	59

[a] Intrinsic viscosity was determined in chloroform at 25 °C; [b] Determined by DSC; n.d.: not detectable; [c] Determined by DMA; [d] Determined by TGA; [e] ΔH°_m = 233.3 J/g [9]; * $T_m = T^\circ_m$ (Hoffman-Weeks equilibrium melting point); # M_w determined using SEC in chloroform at 25 °C; * M_w determined using HT-SEC in TCB at 160 °C.

5.4.1. Thermal properties

In Table 5-3, the thermal properties of PPDL are listed as they have been reported in literature and as determined in this study on high molecular weight PPDL (M_w = 190 kg/mol).

Supporting data for the observations in the present study is presented in Figure 5-6 to 5-8. From Table 5-3, it can be concluded that most values are in the same range. As no data are available on the conversion from intrinsic viscosity to actual molecular weights for PPDL, the data from the first two entries cannot be scaled to the molecular weights in the other entries.

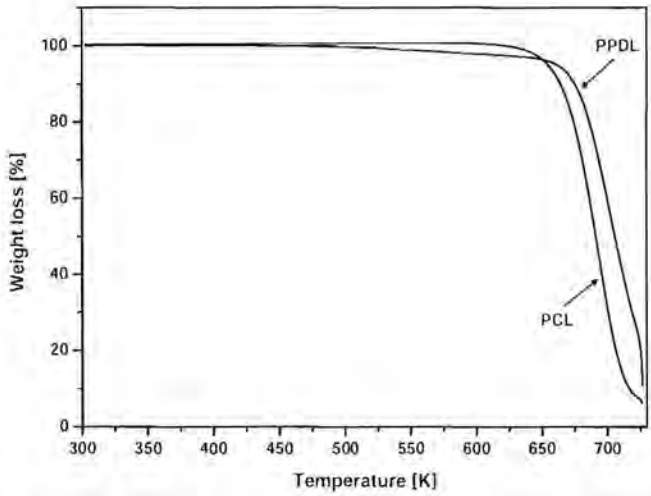

Figure 5-6: *Results of thermogravimetric analysis on PCL and PPDL both synthesized via e-ROP in an inert atmosphere (N_2).*

Figure 5-6 shows the results of thermogravimetric analysis (TGA) for both PCL and PPDL. PCL, used for this TGA-analysis, was synthesized by e-ROP (M_w = 60 kg/mol based on PS-standards). The result sin Figure 5-6 clearly demonstrate that significant degradation of PCL starts at 625 K. PPDL rapidly looses weight at 660 K. Hence, it can be concluded that PPDL has a better thermostability than PCL, and shows similar stability as LDPE.[29] The small weight loss, observed for PPDL at about 525 K, is believed to originate from residual traces of monomer (~2-3 w/w-%). Residual monomer was not determined using ^{1}H-NMR spectroscopy, but it may have given the polymer its distinct smell.

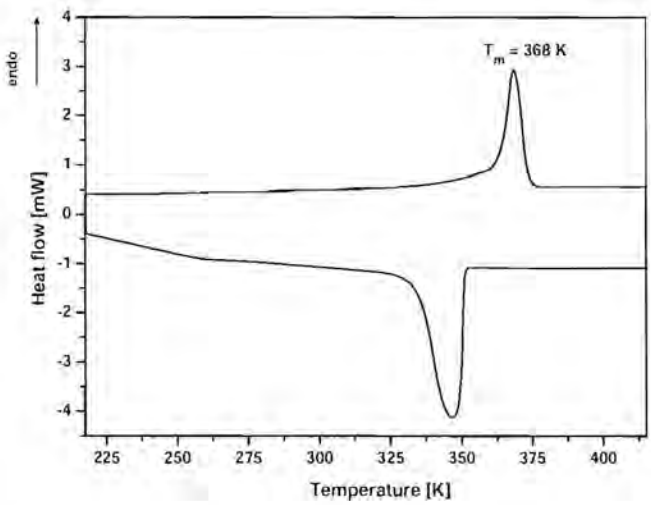

Figure 5-7: *DSC-curve of PPDL: heating rate: 10 K/min; cooling rate: 20 K/min.*

Differential scanning calorimetry (DSC) can provide information about the glass transition temperature, T_g, as well as about the melting and crystallization behavior of (semi-)crystalline polymers. In Figure 5-7, a calorigram of PPDL is depicted. The melting point, T_m, of PPDL can clearly be observed at 368 K, which is very close to the reported literature values. Depending on the cooling rate, crystallization occurs at 346.5 K (20 K/min) to 353.5 K (2.5 K/min). Zhong[6] and Skoglund[15] already reported that PPDL is a rapidly crystallizing polymer. Indeed, even for our high molecular weight PPDL, the required supercooling ($T_m - T_c$) is very small. In agreement with Lebedev[9], the Hoffman-Weeks equilibrium melting point (T^o_m) of 370.5 K was determined. From optical microscopy, a spherulitic morphology was observed, due to a large number of heterogeneities acting as primary nucleating agents. Spherulitic growth rates were too high to be determined. Moreover, the observed lower degree of crystallinity of our high molecular weight PPDL (59%) with respect to previous reports is an additional indication that our polymer has a significant higher molecular weight than the polymers reported in previous publications.

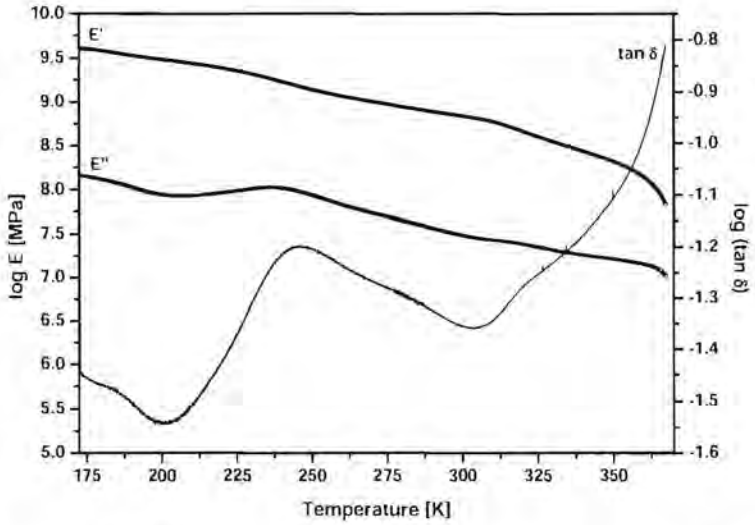

Figure 5-8: *DMA-curve of PPDL; logarithmic storage and loss moduli on left axis and logarithmic tan δ on right axis.*

The glass transition temperature of PPDL could not be determined by DSC in this study, nor in the studies of Zhong[6] and Focarete[13], probably due to the high crystalline fraction of the polymer. Hence dynamic mechanical analysis (DMA) was used to determine the T_g. Figure 5-8 shows the logarithmic loss and storage moduli (E'' and E'), as well as their ratio, tan δ. The local maximum of tan δ represents the α-relaxation of the polymer, which corresponds to the glass transition temperature, T_g[30], which is observed at 248 K. The inflection point at 185 K is believed to correspond to β-relaxations due to polar interactions of the ester bond with water.[13]

5.4.2. Tensile properties

Compression molded films

Mechanical properties of PPDL have hardly been investigated. Only Focarete et al.[13] have reported very briefly on these properties, but for PPDL with a significantly lower molecular weight, as determined by SEC in chloroform at 25 °C. In Table 5-4, the tensile properties are collected as they were observed in the study of Focarete and in the present study.

Table 5-4: *Overview of tensile properties (of compression molded film) reported in literature and discussed in this evaluation.*

Entry	M_w [kg/mol]	M_w/M_n [-]	E [MPa]	σ_{break} [MPa]	σ_{yield} [MPa]	ε_{yield} [%]	ε_{break} [%]
Focarete et al.[13]	129 *	2.0 *	370	-	14.5	12	100-200
present study	190 #	3.2 #	420	38	17.5	15	>1200

PPDL was compression molded and perforated in dumbbells (exact size: see Experimental section); * determined using SEC in chloroform at 25 °C; # determined using HT-SEC in TCB at 160 °C; both samples are calibrated on PS-standards.

Compression molding of PPDL at a maximum temperature of 130 °C was observed to cause a slight color change of the polymer (yellowish). It is not sure whether this is due to monomer residues or other residual fractions originating from enzyme and molecular sieves that were still present in the polymer, or whether it is caused by the polymer itself (not uncommon for aliphatic polyesters). It was investigated whether this color change also occurred under vacuum and 120 °C. No color change was observed, but when vacuum was released at 120 °C with air, rapid color change (yellow to brown) was observed. Thus, it can be assumed that this color change is due to oxidation of the polymer.

The tensile modulus, E, was observed to be around 420 MPa for the compression molded films, which is in the same order as reported in literature (Table 5-4). The stress at break (σ_{break}), which had not been reported before, was observed to be up to 38 MPa, if the used film was free of air bubbles and other defects. Typically, the strain at break of the PPDL synthesized in this study (ε_{break} > 1200 %) was observed to be much higher than had previously been reported (ε_{break} = 100-200 %). Possibly, the low elongation at break as observed by Focarete is caused by small defects in the PPDL-film (air bubbles, etc.), as was also observed in this study (Figure 5-9). Additionally, one should keep in mind that the determination of the molecular weight distribution using SEC in chloroform at 25 °C as applied by Focarete may be misleading as only the lower molecular weight fractions appear to dissolve (Section 5-3). Hence, low molecular weight fractions have most probably been present in the PPDL-polymer studied by Focarete et al. which act as plasticizer and have a deleterious effect on the elongation at break.

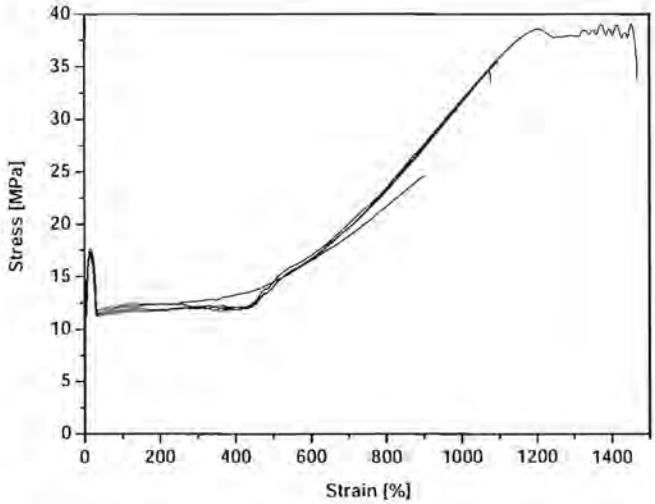

Figure 5-9: *Stress-strain behavior of dumbbells from a compression molded film of PPDL (M_w = 190 kg/mol).*

Fibers

PPDL fibers were spun directly from the melt (at 115 °C), resulting in very non-uniform, fibers with varying diameter. These fibers were stretched to the maximum at 90 °C (~6-8 times the original length) and their tensile properties were tested. Initial results showed that the maximum tenacity for these fibers was ~0.6 GPa. However, since most of the fibers contained defects, a melt flow indexer was applied to obtain more uniform PPDL fibers. Using a 1.05 mm dye and a temperature of 120 °C, PPDL was molten and pushed through the nozzle, such that fibers were obtained. By varying the mass of the weight cell, temperature, and the winding speed (up to 10 m/min), fibers with different elongations and thicknesses were obtained. Elongation of these fibers was applied at 90 °C and after reaching the maximum of 6-8 times the original length, the elongation was continued at 103 °C. Tensile tests on these elongated fibers revealed an average tenacity of ~0.6 GPa.

Wide angle X-ray scattering (WAXS) and DSC-analysis of the latter fibers are currently in progress, thus these results could not be included in this thesis. Initial results from WAXS showed a complete lack of orientation in the original fibers, whereas with increasing elongation, orientation is induced into the PPDL fibers.

5.4.3. Degradation behavior

So far PPDL has always been considered a biodegradable polyester with properties resembling polyethylene. The actual degradability of PPDL however has not been investigated before. In this study, the hydrolytic degradation of PPDL was investigated in order to obtain information on the rate of degradation and to be able to compare the degradation with other aliphatic polyesters. Hydrolytic degradation is believed to occur via bulk erosion, as water can diffuse into the polymer.

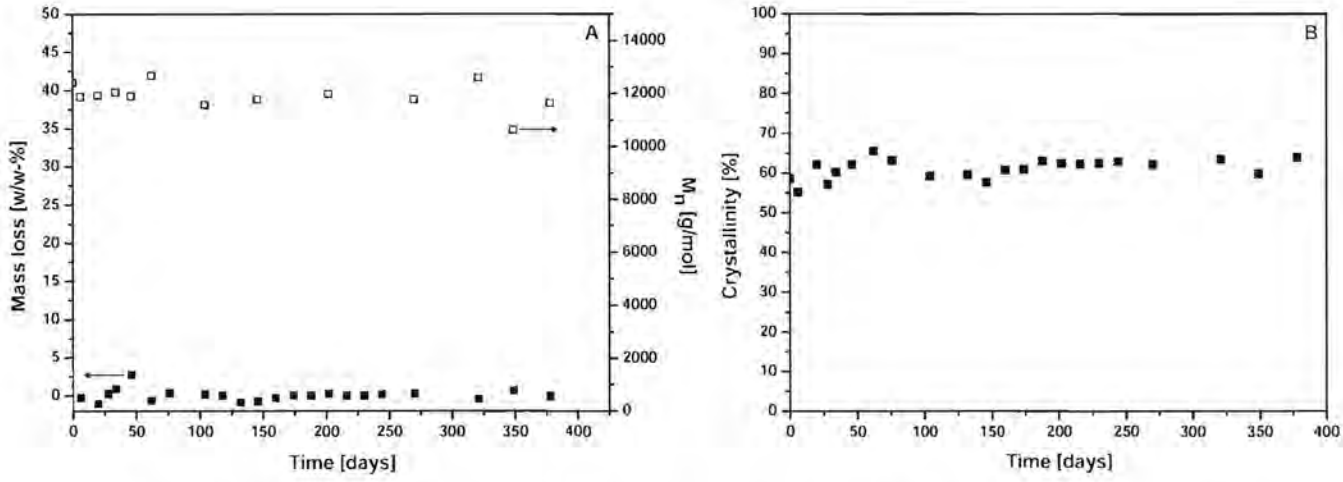

Figure 5-10: *Hydrolytic degradation of PPDL in phosphate-buffer at 37 °C: **A:** mass loss and molecular weight, M_n, as a function of degradation time; **B:** crystallinity of PPDL during degradation; M_n was determined using ^{1}H-NMR and the crystallinity was determined using DSC.*

PPDL (M_n = 12.4 kg/mol) was processed in a melt flow indexer at 110 °C, and a cylindrical shape was obtained with an average diameter of 1.41 mm (± 0.16 mm). Exact weighed amounts of this polymer (~200 mg) were placed in test tubes and phosphate-buffer (~20 mL) was added. Samples were stored in an oven at 37 °C. Figure 5-10A shows the effect of storage at hydrolytic conditions on mass loss and on the number average molecular weight (M_n) of PPDL. No significant degradation of PPDL was observed even after approximately one year (378 days) in a phosphate buffer. The molecular weight of the polymer (determined with ^{1}H-NMR spectroscopy) reveals only a minor decrease (from 12.4 to 11.6 kg/mol), and mass loss did not increase significantly. Moreover, the crystallinity of the polymer showed a slight increase from 58% at the start of the experiment to 64% after one year (Figure 5-10B). Probably, this increase in crystallinity is caused by water, functioning as plasticizer in the polymer, thereby increasing the flexibility of the polymer chains in the amorphous phase.[31] It can be concluded that hydrolytic degradation of PPDL has a long inhibition time and degradation (i.e. decrease in molecular weight) probably starts off more than one year after exposure to an aqueous phosphate buffer solution.

In addition, enzymatic degradation of PPDL at 37 °C was investigated, in order to verify the reactivity of the available ester bonds in PPDL (in the amorphous phase). *Pseudomonas Cepacia Lipase* (PS-lipase), a common enzyme for enzymatic degradation experiments[32], was used in this experiment, and PPDL was prepared under exactly the same conditions as before (M_n = 12.2 kg/mol). In this experiment a lipase was applied to catalyze hydrolysis. Hence, only surface erosion was expected, as the lipase cannot diffuse into the polymer. Figure 5-11 shows the effect of enzymatic hydrolysis of PPDL on mass loss and the number average molecular weight, M_n.

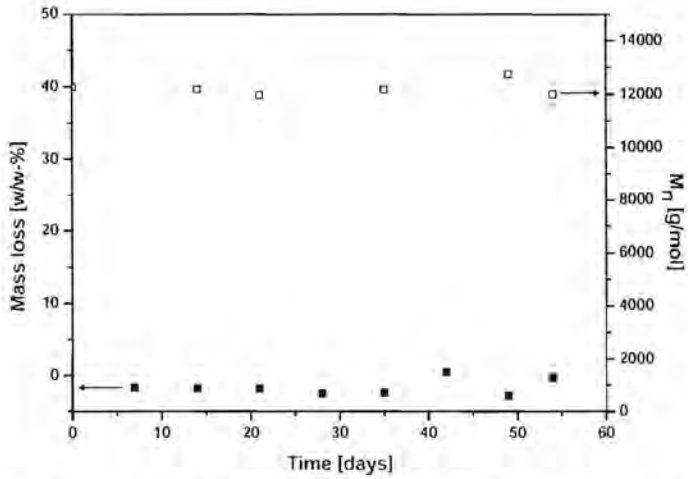

Figure 5-11: *Enzymatic degradation of PPDL in phosphate-buffer in presence of* Pseudomonas Cepacia *lipase at 37 °C; mass loss and molecular weight as a function of degradation time;* M_n *was determined using* ^{1}*H-NMR.*

The results in Figure 5-11 clearly demonstrate that no significant degradation of PPDL was observed after 60 days exposure to a phosphate buffer solution at a temperature of 37 °C in the presence of *Pseudomonas Cepacia* lipase. In the following section (section 5-5), the degradability of PPDL is compared to the degradability of other polymers and further discussed. In conclusion, it can be stated that the PPDL is not as degradable as expected. Probably, the high crystallinity of PPDL and the hydrophobic aliphatic backbone limit accessibility of water onto the ester bonds of the polymer. In addition, the amorphous domains may not be accessible, especially for lipase.

5.5. Positioning PPDL

In the previous section, an overview was given on the current data on PPDL-properties. In this section, all data will be compared with polymers, with which PPDL is believed to be able to compete from a structural point of view, such as polyethylene (PE), poly(ε-caprolactone) (PCL) and poly(L-lactic acid) (PLLA). Since PPDL can only be formed as a linear, non-branched structure, comparison would thus only be appropriate with linear polyethylenes, i.e. LLDPE and HDPE. With respect to the molecular weights, for all three classes of PE molecular weights of 20 to 200 kg/mol relevant data have been reported (HDPE even up to M_n ~1000 kg/mol).[36] Again, this section will be divided in thermal properties, tensile properties and degradation behavior.

Table 5-5: *Thermal properties of PLLA, PCL, PPDL, HDPE, LDPE and LLDPE;*

Polymer	T_m [K]	T_g [K]	T_{degr} [K]	χ_c [%]
P(L)LA	451 [33]	330 [34]	625 [35]	30 [33]
PCL	330 [17]	213 [34]	650 [35]	56 [17]
PPDL	370	250	675	75
LLDPE	395-400 [36]	210-230 [36]	n.d.	30-45 *, [36]
LDPE	370-390 [36]	248 [36]	650 [36]	n.d.
HDPE	400-410 [36]	148 [36]	n.d.	n.d.

* depends on the type and amount of branching; n.d. : no data.

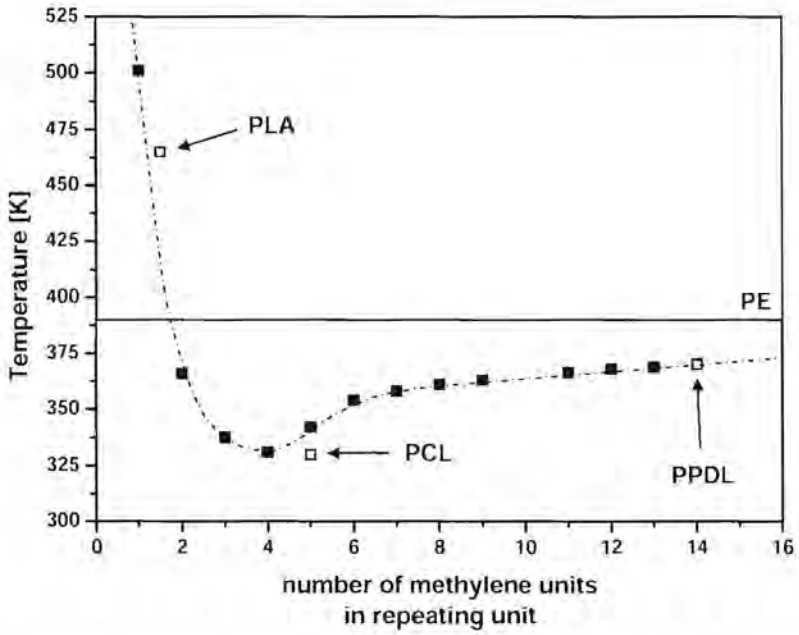

Figure 5-12: *Predicted evolution of the equilibrium melting temperature as a function of the number methylene units in the repeating unit by Lebedev [9]; ■: equilibrium melting temperature, T°_m; □: observed melting temperatures for PPDL, PCL [17] and PLA [33].*

5.5.1. Evaluation of thermal properties

In Table 5-5, the thermal properties of PPDL are collected together with those of PCL, PLA and PE. When comparing the melting temperature, T_m, of the various polymers, the data collected in Table 5-5 demonstrate that the T_m of PPDL approaches the T_m of PE. This observation corresponds to a pattern that was predicted by Lebedev et al[9], who showed a convergent relationship for the equilibrium melting temperature of polyesters (T°_m) as a function of the number of methylene units in the repeating unit of the polymer backbone (Figure 5-12). The observed melting temperatures, T_m, of PPDL, PLA and PCL (values from Table 5-5) are also presented in Figure 5-12, from which it can be concluded that the observed values follow Lebedev's trend. Lebedev predicted a similar relationship between the number

of repeating units in the polymer backbone and the equilibrium glass transition temperature, T°_{g}. The highest reported degree of crystallinity, χ_c, for PPDL is 75%. This value can is much higher than the degree of crystallinity observed in e.g. LLDPE. Here it should be mentioned, that the degree of crystallinity of LLDPE strongly depends on the comonomer that was used, i.e. 1-butene, 1- hexene, or 1-octene. The smaller the side-group, the higher the possible degree of crystallinity.[37]

5.5.2. Evaluation of tensile properties

The data obtained in this study show that PPDL has similar mechanical properties than LDPE (Table 5-6). Low molecular weight fractions will act as an undesired plasticizer, as was reported previously.[13] When comparing the tensile modulus of PPDL with other biomedical polymers, it is observed that PLLA shows far higher tensile modulus in a compression molded film, since at room temperature the polymer is in its glassy state. PLLA would clearly be the preferred polymer for high-strength applications such as orthopaedic implants (e.g. degradable bone nails). However, the high tensile modulus comes with extreme brittleness of PLLA (ε_{break} ~7%). PCL and PPDL are rather similar in terms of tensile strength and modulus.

Table 5-6: *Mechanical properties of compression molded films of PLLA, PCL, PPDL and PE.*

Polymer	E [MPa]	σ_{break} [MPa]	σ_{yield} [MPa]	ε_{yield} [%]	ε_{break} [%]
PLLA	1000-3000 [33]	n.d.	50 [33]	<4 [33]	<7 [33]
PCL	400 [33]	n.d.	16 [33]	7.0 [33]	800-1000 [33]
PPDL	420	38	17.5	15	1200
LLDPE	250-500 [36]	13-27 [36]	9-19 [36]	n.d.	100-965 [36]
LDPE	200-300 [36]	8-30 [36]	9-14 [36]	n.d.	100-650 [36]
HDPE	1100 [36]	21-32 [36]	n.d.	n.d.	1200 [36]

n.d.: no data.

5.5.3. Evaluation of degradation behavior

In advance PPDL was expected to show slow hydrolytic degradation, as PCL and PLLA are known to need more than two years for complete mass loss.[38] Nevertheless, PPDL did not show any significant degradation within one year, probably due to its high crystallinity and its hydrophobic character. The molecular weight of PPDL used in the degradation experiments was too low for fiber applications (M_n = 12.4 and M_n = 12.2 kg/mol for hydrolytic and enzymatic degradation, respectively). Hence, it can be expected that when applying high

molecular weight PPDL fibers for biomedical applications, these fibers will maintain their mechanical properties for long implantation times.

In order to make PPDL faster degradable, random copolymers could be synthesized, thereby reducing the crystallinity, and depending on the comonomer, introducing more hydrophilic segments into the polymer backbone.[39] When PDL was copolymerized with 4-methyl caprolactone (4MC) (M_n = 29 kg/mol), preliminary results of hydrolytic degradation of the random copolymer (poly(4MC-*co*-PPDL)) showed an immediate decrease of the molecular weight.[40] Nevertheless, after one year, no mass loss had been observed yet. This observation confirms the assumption that chain cleavage occurs mainly in the amorphous domains, and hydrolytic degradation of aliphatic polyesters is a slow process. However, for fiber properties the crystalline domains provide strength to the material. Hence, the lateral strength needed for fiber application and fast degradability seem to be two conflicting material properties, which makes the PPDL fibers perfectly applicable for long term use.

The *in vivo* degradation behavior of PPDL was not examined, and these results would give the ultimate answer to the question whether PPDL is degradable or not. In this *in vivo* study, the effect of the degradation products should be carefully studied, as the final degradation product, ω-hydroxy pentadecanoic acid, has been reported to be a carcinostatic compound*, probably due to the hydrophilic/hydrophobic balance of the molecule.[41] An additional advantage of the degradation of PPDL is the low acidity of the degradation products when comparing to PLA, from which it is known that the high acidity of its degradation products usually causes inflammation.

5.5.4. Possible applications for PPDL

In this chapter different properties of PPDL were assessed in order to determine the scope and possibilities of this novel polyethylene-like polyester. PPDL is an aliphatic polyester with thermal properties close to those of LDPE (Table 5-5) and mechanical properties resembling those of PCL and LLDPE (Table 5-6) with possible degradability. ω-Pentadecalactone (PDL) is a chemical that is currently used by fragrance industry as a musk odor in perfumery and as odorant in cigarette filters. Industrial synthesis of PDL is normally achieved via ring-expansion reactions from cyclododecanone (three step synthesis).[42] Although it is one of the least expensive macrocyclic musk compounds, the unit price of the monomer is too high, which excludes the application of PPDL as a bulk polymer, such as polyethylene, polypropylene, and polycarbonates. In general, the unit price of biodegradable polymers is the main constraint on their use when compared to bulk produced oil-based plastics.[43]

In addition, PPDL could be applied for biomedical purposes, as unit prices are much less important than the *in vivo* performances in these applications. Typical biomedical applications

* Carcinostatic = *pertaining to slowing or stopping the growth of cancer (www.cancer.gov).*

include suturing, orthopaedic devices, controlled drug delivery, stents, and tissue engineering.[3] PPDL-fibers and tapes can be interesting materials for internal suturing and bone repair, especially when long time healing processes are involved. As the crystallinity of PPDL and its hydrophobic character seem to retard the degradation process, copolymers of PPDL with other polyesters can be considered when fast degradation is desired. By incorporating hydrophilic building blocks such as glycolide, lactide, 1,4-dioxanone (DXO), and new monomers such as 2-oxo-12-crown-4-ether[23] randomly, the hydrophilic character of the copolymer improved, but simultaneously, the crystallinity of the copolymer is reduced causing reduced strength of the obtained material. Moreover, interesting block copolymers can be produced comprising hydrophilic and hydrophobic blocks in one molecule, e.g. with polyethylene glycol (PEG). Hence, the ester-moiety in PPDL allows this polymer covalent bonding with different building blocks, from which novel materials can be produced.

5.6. Conclusions

The enzymatic ring-opening (e-ROP) of ω-pentadecalactone (PDL) was investigated and compared to e-ROP of ε-caprolactone (ε-CL). The enzyme, Novozym 435, showed higher activity towards PDL than to ε-CL and this was confirmed in literature. Enzymatic polymerization of PDL showed a remarkably fast conversion rate (>90% PDL conversion in 15 mins at 60 °C) and PPDL turned out to be very sparsely soluble at room temperature, when molecular weight increased. This poor solubility resulted in complications with regard to analysis, especially in size exclusion chromatography (SEC). Although in literature reports SEC in chloroform at 25 °C is always used (for M_n up to 200 kg/mol), a pressure increase was observed when performing this analysis on PPDL. This probably indicates that the polymer was not completely dissolved in the eluent. Hence, we performed SEC in 1,2,4-trichlorobenzene at 160 °C, which showed more accurate and reproducible results. This latter analysis revealed a high fraction of low molecular weight material in the PPDL samples, whereas this had not been observed when using SEC in chloroform. Therefore, any data on molecular weights of PPDL determined with SEC in chloroform should be considered with great care.

High molecular weight PPDL could be obtained when several conditions were met: (i) the use of an appropriate overhead stirrer that is designed for mixing highly viscous solutions, (ii) reaction temperature of 85 °C to prevent crystallization of PPDL during the reaction, (iii) thorough drying of the system, reactants and enzyme, (iv) the use of molecular sieves in the reaction mixture, and (v) the use of para-oxon to instantly terminate the reaction. The highest obtained weight average molecular weight, M_w, was 91 kg/mol with a corresponding polydispersity index of 2.9 (based on calibration on PE-standards) with a yield of almost 30

grams. This roughly corresponds to a M_w of 190 kg/mol and a corresponding polydispersity index of 3.2, based on PS-standards (transformation required for literature comparison).

This high molecular weight PPDL was further investigated to reveal its thermal and mechanical properties as well as its hydrolytic degradation behavior, to allow a comparison of these properties with data reported earlier, and subsequently to compare these data with those of other polymers such as polyethylene, poly(ε-caprolactone) and poly(L-lactide). In conclusion, the thermal properties were similar to LDPE with high crystallinity (T_m: 97 °C; T_g: -25 °C; χ_c: 60%). With respect to mechanical properties, a compression molded PPDL film is positioned between PCL and HDPE (E = 420 MPa; ε_{break} ~1200 MPa). In addition, the degradation behavior was investigated for the first time. Due to its high crystalline fractions and its highly hydrophobic character, PPDL did not show significant hydrolytic degradation after one year in an aqueous phosphate buffer solution.

5.7. Experimental Section

Materials

All chemicals were purchased from Aldrich, stored over molecular sieves and used without further purification unless otherwise noted. Toluene (Biosolve, AR-grade) was dried over alumina and stored over molecular sieves. Novozym 435 was obtained from Novozymes A/S and stored over P_2O_5 in a desiccator. Molecular sieves (3Å) were dried in an oven at 420 °C prior to use. Para-oxon was dissolved in toluene before use as enzyme-inhibitor to instantly stop the reaction.

Methods

^{1}H-NMR spectroscopy was performed using a VARIAN 400 NMR at 25 °C with $CDCl_3$ as solvent and TMS as internal standard. Data were acquired using VNMR-software. The molecular weight distribution of the polymers was determined by size exclusion chromatography (SEC) in chloroform at 25 °C on a PLgel 500 Å column (30 cm×7.5 mm I.D., 5 μm particles, Mixed D) from Polymer Labs. The measurement system consisted of an LC-10AT pump operating at 1 mL/min and a Refractive Index Detector (RID) (Shimadzu, Kyoto, Japan). Therefore, all samples were diluted to 1.0 mg/mL in chloroform. All molecular weights were determined using a PS-calibration. SEC-analysis in 1,2,4-trichlorobenzene (TCB) was performed on a 3*PLgel mixedB 10 μm + PLgel 10 μm column set with DRI-detection. TCB was used as eluent with a flow rate of 1.0 mL/min. All samples were diluted to 0.10 mg/mL in TCB. All molecular weights were determined using PS-calibration. SEC-analysis on a high throughput SEC was performed using a PL-Rapide H 105*7.5mm-column at 160 °C. TCB was used as eluent with a flow rate of 1.0 mL/min. Molecular weights of

PPDL and PCL were calculated based on PE-standards and can be converted to molecular weights on PS-standards by using equation 5.2.

Thermogravimetric analysis (TGA) was performed on a Perkin Elmer Pyris 7 TGA with N_2 as purge gas. Approx. 5 mg of polymer was weighed into porcelain cups. The applied temperature profile was a heating rate of 5 K/min from 300 to 725 K. Data were processed with TA-Universal Analysis software. Differential Scanning Calorimetry was performed on a TA Q100 DSC. Approx. 5 mg of dried polymer was weighed into aluminum hermetic pans. Temperature profiles from 213 K to 423 K with a heating rate of 10 K/min and cooling rates of 2.5, 10 and 20 K/min were applied. TA Universal Analysis software was used for data acquisition. Compression molded films were prepared using a press, by heating the material at 130 °C for 20 minutes and a pressure of ~150 bar. The films were rapidly quenched to room temperature with water. Dynamic mechanical analysis (DMA) was performed on a TA DMA Q800 V5.1 with dual cantilever clamp. Data was acquired using TA Universal Analysis. Compression molded bars of approx. 14.4×5.4×1.2 mm size were prepared and clamped in. A temperature profile from 173 K to 368 K with a heating rate of 1 K/min and a frequency sweep of 1 Hz was applied. TA Universal Analysis v4.1D software was used for data acquisition. Tensile tests were performed on a Zwick tensile meter with a 10 kN load cell on compression molded films that were perforated in dumbbell shapes (approx. 16×4.9×1.2 mm) with a tensile speed of 10 mm/min. Data acquisition was performed with TestXpert V8.1 software.

The hydrolytic degradation experiment was performed on cylindrical rods (diameter: ~1.4 mm) of PPDL. Approx. 200 mg was accurately weighed in test-tubes, followed by the addition of a phosphate buffer solution (20 mL). The tubes were stored in an oven at 37 °C. After predetermined time intervals, samples were removed from the oven, the phosphate buffer was removed and the residual polymer washed with fresh water. The samples were dried in a vacuum oven at room temperature to remove the water. Subsequently, mass loss was determined on a balance, and the molecular weight was determined with ^{1}H-NMR spectroscopy. Crystallinity was determined with DSC. For the enzymatic degradation of PPDL, similar rods were used. A phosphate buffer solution was used and per 10 mL of solution, 3 mg of *Pseudomonas Cepacia Lipase* (Amano, powder) was added. The degradation experiment followed the same procedure as the hydrolytic degradation.

Enzymatic polymerization of PDL with benzyl alcohol as initiator

For all enzymatic polymerizations, the amount of Novozym 435 with respect to monomer was kept constant at 10 w/w-% unless stated otherwise. Prior to the reaction, 0.297 g of Novozym 435 was weighed into the reaction flask. Subsequently, the flask was heated in an oil bath to 60 °C. The reaction was started by adding a stock solution containing 33 mg (0.3 mmol) of benzyl alcohol, 2.911 g (12.11 mmol) of PDL and 5.560 g (60.34 mmol) of toluene

to the flask. At specified time intervals samples (~0.1 mL) were withdrawn from the reaction mixture with a syringe and analyzed by ^{1}H-NMR spectroscopy to determine the monomer conversion.

Enzymatic synthesis of high molecular weight PPDL (laboratory scale)

In a typical enzymatic polymerization of PDL, Novozym 435 (10 w/w-% to monomer, 0.249 g) was dried in a 10 mL flask with molecular sieves (3Å) and a magnetic stirringr bar under vacuum at 50 °C overnight. After drying, the flask was removed from the oven under nitrogen atmosphere and it was closed with a septum. The system was heated up to 70 °C with an oil bath. When a stock solution of ω-pentadecalactone (PDL, 2.498 g, 10.41 mmol) and toluene (5.021 g, 54.49 mmol) had been added to the enzyme through the septum, the reaction started and the stirring was started. The reaction was ended after 24 hours by the addition of para-oxon and filtration of the immobilized enzyme and molecular sieves after dilution with hot chloroform. The polymer yield was 1.978 g (79%). ε-Caprolactone (ε-CL) was polymerized according to the same procedure, but purification was performed using dichloromethane at room temperature.

Up-scaled enzymatic synthesis of high molecular weight PPDL

In order to upscale this polymerization for property testing, a larger setup was used, consisting of a double-walled glass vessel (300 mL). First, Novozym 435 (1 w/w-% to monomer 0.296 g) was dried in a glass vial at 50 °C under vacuum for 16 hours (standard drying method)[30] and the reaction vessel was dried in an oven at 150 °C together with molecular sieves. Subsequently, the vacuum was released using N_2 and it was placed in the reaction vessel, equipped with a metal overhead stirrer, and brought under argon atmosphere. Then, the vessel was connected to an oil bath and heated to 85 °C. A stock solution of PDL (33.692 g) and toluene (29.603 g) was added to the vessel using a preheated glass syringe and needle and the reaction started (stirrer-speed: 30-50 rpm). After 72 hours, a highly viscous slurry of enzyme and PPDL in toluene had been obtained and the reaction was stopped by adding a mixture of para-oxon in toluene (35.5 mg in 15 g of toluene) in double excess to the actual amount of enzyme as inhibitor[31], followed by 150 mL of preheated p-xylene (100 °C) to dissolve the polymer. The slurry was stirred for 1 hour and subsequently the enzyme was carefully filtrated over a Buchner-funnel using an additional amount of preheated p-xylene. p-Xylene was partially removed by evaporation (until ~400 mL was left) and then methanol (200 mL) was added at 60 °C to neutralize para-oxon. By further reducing the temperature to 20 °C the polymer precipitated and was filtrated over a Buchner-funnel. Finally, the polymer was dried under vacuum overnight at 50 °C, and 28.65 g of PPDL-product was obtained.

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6

Technology Assessment & Outlook

Abstract

In this chapter, industrial up-scaling of the chemoenzymatic cascade process discussed in Chapter 4 is evaluated, based on the choice of reactor, reactor design, and requirements that would have to be met when implementing such a system. In addition, the future prospect for the role of enzymes in polymer chemistry is discussed.

6.1. Industrial application of enzymatic ring-opening polymerization

In this work the polymer syntheses described were all based on batch processes. Currently, only batch operated stirred tank reactors are applied for industrial enzyme-catalyzed processes, due to the (relatively) small volumes produced per batch, and the versatility towards changes in the production.[1-3] Implementation of an enzymatic polymerization for the large scale production of polymers demands for a continuous process, since the use of (immobilized) enzymes in a batch process has some specific drawbacks, e.g. a more complicated regeneration of the immobilized enzyme due to the viscosity increase during polymer production. However, the industrial production of polymers in a continuous process would only be economically feasible if large volumes are produced. In this section, the feasibility of enzymatic ring-opening polymerization into a continuous process is discussed.

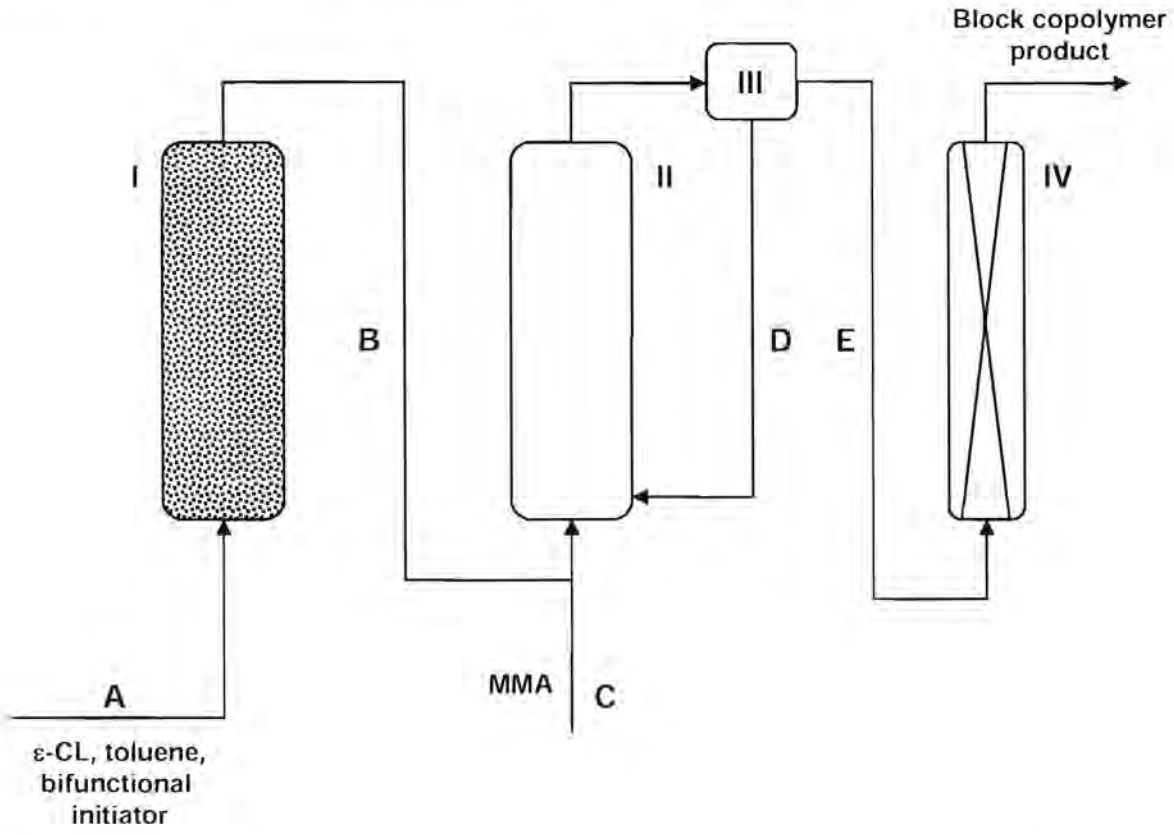

Figure 6-1: *Reaction scheme for chemoenzymatic cascade synthesis of poly(CL-b-MMA) in a continuous system; A: monomer feed; B: product flow; C: comonomer feed; D: ATRP-catalyst regeneration; E: product flow; I: Tubular reactor filled with immobilized enzyme on porous particles (e.g. Novozym 435); II: Tubular reactor for ATRP; III: ion exchanger for ATRP-catalyst regeneration; IV: solvent stripping.*

The chemoenzymatic cascade synthesis of a block copolymer (see Chapter 4) would be an excellent example for industrialization of an enzymatic polymerization, as the time-scale in which both polymerizations take place (i.e. e-ROP of ε-CL and ATRP of MMA) is in the

same order of magnitude. The block copolymer produced could be applied as compatibilizer. Figure 6-1 shows a schematic representation for the chemoenzymatic cascade synthesis of poly(CL-*b*-MMA) based on the requirements and limitations observed in the laboratory-experiments discussed in Chapters 2 to 4.

The design of the continuous reactor is based on two tubular reactors in series (**I** and **II**). For plug flow in these tubular reactors, complete monomer conversion in the exit flow is possible. A continuous stirred tank reactor (CSTR) is not a proper reactor of choice since conversions are low, and molecular weight distributions will be broad as a result of residence time distribution. It was observed that the transition metal used in ATRP had a deactivating effect on the enzyme, due to coordination of the metal by the enzyme (see Chapter 4). Hence, the two catalysts, i.e. enzyme and CuBr/ligand, must be completely separated in the process, which is only possible in a consecutive process. Only then, the regeneration of the ATRP-catalyst is well possible and the life-time of both catalysts may be sufficiently long. Since enzymatic ring-opening polymerization e-ROP was observed to barely work from a macro-initiator, first the enzymatic polymerization should be performed from a bifunctional initiator (**I**), followed by the ATRP of a (meth)acrylate (e.g. MMA) from the PCL macro-initiator (**II**).

Immobilized enzymes have proven to be ideal biocatalysts when applied in a tubular reactor, e.g. for simple hydrolysis and racemization reactions.[4, 5] However, with respect to enzyme-catalyzed polymer production in a tubular reactor several intrinsic parameters must be taken into account. Upon polymerization, the molecular weight of the polymer will increase, causing a dramatic increase of the viscosity. Therefore, mass transport may become limiting and the pressure drop in the reactor will become (too) large. This may eventually lead to lack of control over the polymerization, resulting in a broader molecular weight distribution, incomplete monomer conversion and the formation of undesired polymer species. To overcome the increase of viscosity, the addition of a solvent that is compatible with both reactions can be considered, e.g. toluene. The pressure drop in the tubular reactor can also be reduced by using larger enzyme support particles (up to 5-10 mm diameter). As Novozym 435 is a shell catalyst (the enzyme is located in the outer 80 μm of the support), an increase of the diameter of the support will not directly imply a variation in intra-particle mass transfer. Only the amount of enzyme per volume unit will be reduced, and hence a longer column is needed. Ultimately, a structured packing could be designed. The enzyme can then be immobilized in a sheet of porous support material fixed on the packing surface. Moreover, to make the process feasible, the catalyst must be stable for many reaction cycles. Deactivation of the catalyst would imply incomplete monomer conversion. Catalyst deactivation makes extra purification of the reaction products necessary. Hence, the use of enzyme particles in a continuous reactor requires more specific investigations towards the (retained) activity of enzymes on immobilized supports.

Finally, to produce pure PCL macro-initiator in the first step, side-reactions in e-ROP must be avoided. Hence, the monomer concentration must be high to avoid cyclic polymer formation, and the water concentration must be minimized. This latter condition may be the most difficult requirement in the design, due to the contradictory function of water, i.e. retaining enzyme activity versus nucleophilic competition resulting in side-product formation (as discussed in Chapter 2). A suggestion to overcome this dilemma may be the use of untreated, highly active enzyme which allows the excess of water to chemically react with monomer before the actual macro-initiator production. After reaching a steady state in which no more water is used as nucleophile, the use of a bifunctional initiator gives optimal macro-initiator production.

Assuming that this procedure allows the production of pure PCL macro-initiator, the product stream and the comonomer are mixed with the homogeneous ATRP-catalyst. This homogeneous mixture of the PCL macro-initiator, the comonomer and the ATRP-catalyst is fed to a second column (**II**). It is of crucial importance that this comonomer and the PCL macro-initiator are miscible, to obtain a very short initiation time. Again, the design of this reactor must be carefully chosen to obtain an optimal residence time to achieve complete monomer conversion. Moreover, as the rate of polymerization in ATRP reactions is governed by the activation/deactivation-equilibrium of the catalyst, accurate information on this aspect of the controlled ATRP reaction is required.

The increase in viscosity is assumed to be less dominant than in the first step (e-ROP), due to the use of the PCL macro-initiator. In addition, pressure drop in the tubular reactor and mass transfer limitations are not an issue when using homogeneous catalysts. However, in spite of the absence of mass transfer limitations and pressure drop problems, one should keep in mind that the regeneration of this catalyst system might not be so straightforward. Transition metals such as Cu^{+} can be regenerated over an ion exchanger (**III**), but the regeneration of the organic ligand will be more complicated and expensive. Moreover, solvent and residual monomer removal is also required after this second reaction step (**IV**). Additionally, attention should be paid to heat transfer during the ATRP-reaction, as the activation/deactivation is influenced by temperature. Structured packing may be used to improve heat transfer to the reactor wall.

In addition to the production of block copolymers, e-ROP could also be applied for the production of high molecular weight PPDL for fiber application (see Chapter 5). The prerequisites that were mentioned in the previously described continuous production of block copolymers are similar, i.e. the use of a tubular reactor with plug flow, studies towards optimization of the immobilized enzyme, and avoiding the formation of (low molecular weight) side-products. However, due to the desired high molecular weights, the increase of viscosity will be much more pronounced. Therefore, when produced in a tubular reactor, the process will require an enormous pump power to overcome the pressure drop in the reactor

system. Additionally, long residence times are required to produce high molecular weight polymer, since it was observed that in a batch process reaction times up to 70 hours were applied (Chapter 5). Because the amount of enzyme per unit volume is much higher in a continuous reactor than in a batch reactor, residence times will be much lower than 70 hours. A possible set-up may be to place several tubular reactors in series, each with reactor with its own pump-unit (Figure 6-2). On the contrary, the production of high molecular weight PPDL can also be performed in a batch operated stirred tank reactor, since the amounts of polymer needed for fiber application are typically very low. In that case, the demands for stirring will be high, in order to prevent grinding of the enzyme and overcoming heat and mass transfer limitation due to high viscosity. A double helical ribbon impeller may overcome all these problems. In addition, the recyclability of the immobilized enzyme will be poor, and cleaning of the reactor requires high temperature and the use of additional solvent.

In conclusion, the implementation of e-ROP into a continuous process will need further attention, especially with respect to the regeneration of the enzyme and the most optimal immobilization. Additionally, the level of side-products formed in the enzymatic polymerization should be carefully monitored to avoid the formation of too much 'dead' material (cyclic polymer and linear water-initiated polymer species). Both ATRP and e-ROP allow a range of monomers, which implies that the proposed continuous process is promising for a variety of possible products.

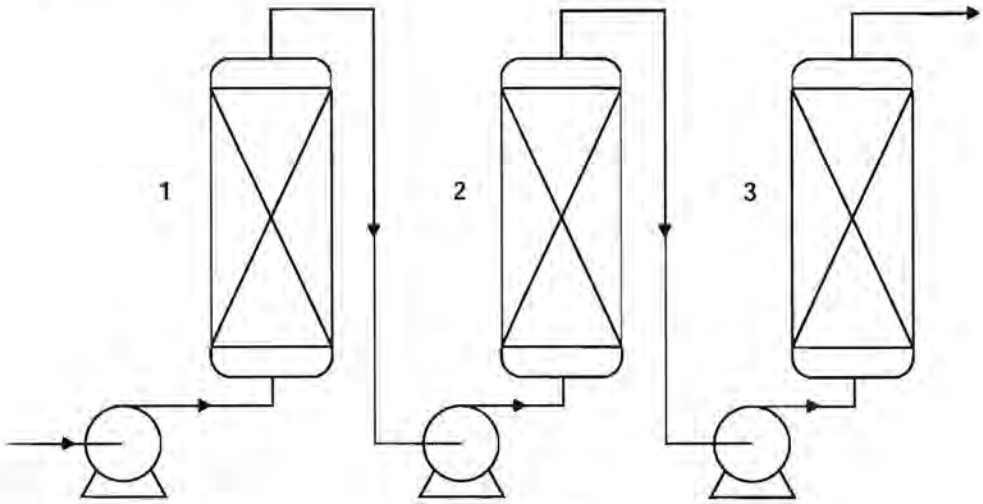

Figure 6-2: *Reaction scheme for enzymatic synthesis of high molecular weight poly(ω-pentadecalactone) (PPDL) in continuous tubular reactors in series.*

6.2. *Prospect of enzymatic polymerization*

Current status

In this thesis, two aspects of the application of enzymes in polymer chemistry were addressed, first the requirements with respect to the implementation of enzymes as catalysts for ring-opening polymerization (e-ROP), and secondly the added value of enzymes in the field of polymer chemistry. This first aspect was extensively studied, resulting in a more detailed insight into the key-steps of e-ROP. With these results better control over the obtained end-products can be obtained in enzymatic polymerization reactions. The added value of enzymes has been discussed before and includes:

- a high activity, which allows reaction under mild conditions,
- the versatility which allows them to work under non-natural circumstances, e.g. in organic solvents and at higher temperature (up to 125 °C),
- chemo-, regio- and enantioselectivity,
- biocompatibility, which makes them ideal for the synthesis of polymers for biomedical *in vivo* applications,
- the ability to be regenerated.

Currently, the main research interests with respect to enzymatic polymerizations can be divided into the application of the produced polymers and the novel synthetic possibilities that enzymatic polymerizations provide. The enzymatic syntheses of biomedical and biodegradable polymers are the main applications of interest, e.g. to avoid the use of usually toxic chemical catalysts, or to introduce specific functional groups that enhance biocompatibility or -degradability. Examples of these include (co-)polymers from 1,4-dioxanone (DXO), ε-caprolactone (ε-CL), trimethylene carbonate (TMC), and ω-pentadecalactone (PDL).

On the other hand, enzymes can also be applied to synthesize novel polymers and polymer architectures. One example of these includes the exceptional activity of enzymes towards macrolides such as PDL, enabling the synthesis of PPDL with exceptionally high molecular weight when compared to the synthesis with chemical catalysts. High molecular weight PPDL seems to be a promising material for biocompatible fibers, although biodegradability should be improved by copolymerization with less hydrophobic comonomers. Moreover, enzymes can ideally be used to reduce the number of reaction steps, also in polymer synthesis. For example, chirality can be introduced in end products starting from a racemic mixture, resulting in optically active moieties and possibly induced crystallinity, without the need of chiral separation of the monomers. Finally, enzymes have been used to make polysaccharides and other sugar-derivates, with high regioselectivity. This would be impossible to achieve using chemical catalysts.

Future aspects

Research towards enzymatic polymerizations was started about 15 years ago, and so far it has predominantly been the domain of organic and polymer chemists. Consequently, this research has primarily focused on kinetic issues and the materials obtained using only a few commercially available enzymes. In Nature enzymes evolved to catalyze one specific reaction. Also for many organic and polymer syntheses, reaction conditions and catalysts have been optimized. On the contrary, no research has so far been performed towards the modification of enzymes for one specific monomer substrate or polymer. Hence, future breakthroughs may be expected from multidisciplinary approaches in which enzymologists are collaborating with organic and polymer chemists, creating the optimal enzyme conformation and reaction conditions for specific monomers and polymers.

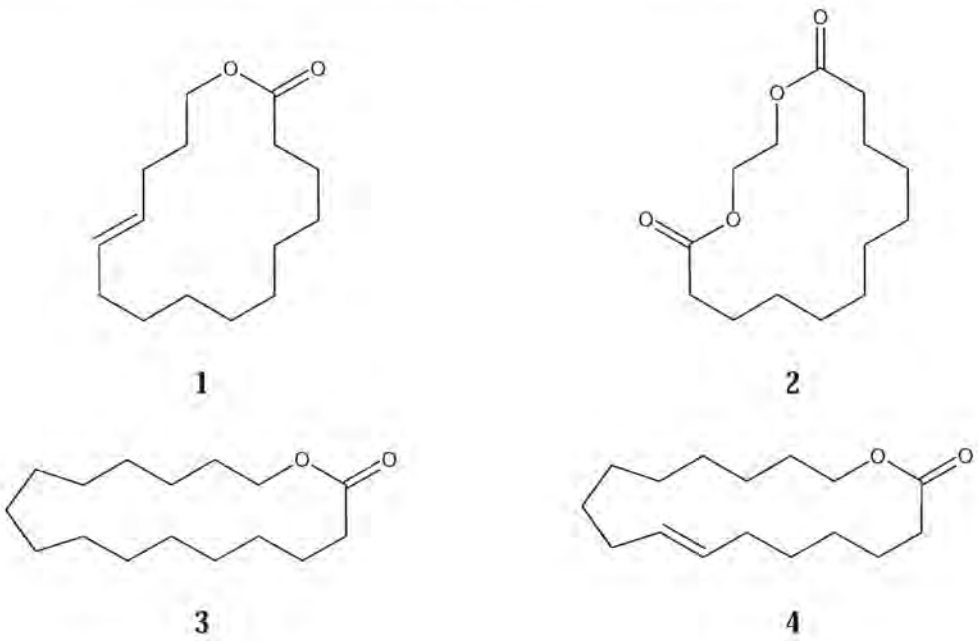

Figure 6-3: *New macrolides available for polymer synthesis (all commercially available and used in perfume industry); **1**: Globalide® - oxacyclohexadecen-2-one; **2**: 1,4-dioxacyclohexadecane-5,16-dione; **3**: hexadecane(16)olide; **4**: oxacycloheptadecen-2-one.*

Figure 6-3 shows different macrolides that can be applied as monomer in e-ROP. By introducing these macrolides as comonomers in polymerizations of PDL or CL, the crystallinity of the polymer can be reduced, thereby enhancing the biodegradation. Also polymer networks can be obtained via the unsaturated moieties in Globalide® (**1**) and oxacycloheptadecen-2-one (**4**).

Finally, by studying polymer synthesis *in vivo*, e.g. the formation of poly(hydroxy-alkanoates) in bacteria, the production of spider silk, or the formation of collagen, starch and DNA, researchers have obtained new insights into precision synthesis via metabolisms. This metabolistic approach reflects effective and advantageous production and may be considered

to be the Holy Grail in synthesis of complex molecules. On the long term these approaches can lead to industrial processes with effective use of chemicals and energy, and ultimately based on renewable feedstock. Although we are only at the early stages of these developments, it can be concluded that enzymatic processes in polymer chemistry have a very promising future.

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Summary

Over the last decade, progress in the research towards enzymatic ring-opening polymerization has lead to novel, biocatalytic, and cleaner processes for the synthesis of polymeric materials. So far, this research has predominantly been focused on how to utilize the enzyme's selectivity to synthesize and modify polymers, which cannot easily be achieved via chemical routes. However, proper understanding of these processes has not been obtained yet. Additionally, already present polymers have been enzymatically synthesized for biomedical applications, without the use of a metal catalyst, e.g. poly(ε-caprolactone). However, only little reports have been published on new materials, which were not readily accessible via traditional polymerization techniques. In this PhD research, the implementation of enzymes into polymer chemistry has been investigated, looking for an answer to the question: Can enzymes open new perspectives in polymer chemistry? Lipase was chosen as the enzyme for enzymatic ring-opening polymerization (e-ROP), as this is well-known in organic synthesis. The lipase that was used in this research is *Candida antarctica* Lipase B immobilized on an acrylic resin, which is commercially available under the name Novozym 435™. The aim of this investigation is to (*i*) obtain insight into the critical parameters of e-ROP of lactones, and (*ii*) make new materials that are not (directly) accessible via chemical polymerization methods.

In order to study the critical parameters of e-ROP of lactones, the present knowledge was investigated in more depth for ε-caprolactone (ε-CL) as monomer in the enzymatic synthesis of end-functionalized polymer using a functional initiator. It was found that only by optimizing reaction conditions such as temperature, presence of water, monomer concentration, and the type of initiator, well-defined polymeric structures could be obtained, thereby limiting the amount of polymeric side-products (i.e. polymer species that lack the specific end-group functionality). Moreover, the concentrations of end-functionalized polymer and the undesired side-products were quantified for the first time using Liquid Chromatography under Critical Conditions (LCCC). This technique has provided us new insights into the actual enzymatic process at different stages in the polymerization. Water appears to be the primary nucleophile during the initial stages of the reaction, even when all the reaction components are thoroughly dried. Depending on the type of functional initiator that is applied, this nucleophile is incorporated into the polymer, by both transesterification and initiation. Finally, cyclic polymer structures are formed during all stages of the reaction and their concentration depends strongly on the initial monomer concentration.

Subsequently, e-ROP was used in combination with controlled radical polymerization (atom transfer radical polymerization, ATRP) in order to investigate the compatibility of enzymes with other catalyst systems. All information that was previously obtained was used to synthesize a block copolymer (poly(CL-*block*-MMA)) by these two polymerization techniques. Ultimately, a cascade chemo-enzymatic polymerization was performed in which the two polymerization techniques were applied simultaneously from a bifunctional initiator to obtain a block copolymer. It was observed that enzymes are slowly deactivated in the presence of transition metals (i.e. copper- and nickel-based ATRP-catalysts), depending on the ligands used to coordinate these metals. Hence, cascade chemo-enzymatic synthesis is only feasible when the two catalysts are applied separately.

In order to synthesize novel materials that are not (directly) accessible via chemical polymerization methods, a larger lactone (i.e. ω-pentadecalactone, PDL) was polymerized using enzymatic ring-opening polymerization. Using chemical, metal-based catalysts, larger lactones cannot be polymerized to high molecular weight polyesters due to their low ring-strain, whereas enzymes have shown surprisingly high activity towards these monomers. The synthesis of this type of monomers opens a novel promising route to the production of biomedical materials. To test the mechanical properties of PPDL, the enzymatic synthesis was scaled up to obtain 30 g of polymer in one batch and optimized to obtain a polymer with a high molecular weight and a relatively narrow molecular weight distribution. The obtained PPDL was melt-spun into fibers, which after drawing show good properties for biomedical applications.

In conclusion, it can be stated that enzymes do open new perspectives in polymer science. Careful analysis of the enzymatic process has revealed the critical parameters for proper enzymatic polymer synthesis. Enzymes can be used in combination with other catalysts and polymerization techniques to make polymer architectures that may not be directly available via chemical synthesis. Finally, a new range of monomers can be utilized specifically by enzymes to make novel, tailored biomedical materials.

Samenvatting

In de huidige samenleving worden thema's als 'natuur' en 'het milieu' steeds belangrijker. Ook de chemische industrie draagt hieraan bij door processen schoner te maken of energie-zuiniger te laten verlopen. Bovendien worden bestaande producten, zoals verpakkings-materialen, steeds vaker vervangen door nieuwe, minder schadelijke of zelfs afbreekbare varianten. Enzymen zijn natuurlijke katalysatoren die ingewikkelde biochemische reacties bij milde omstandigheden mogelijk maken. Daarom worden enzymen de afgelopen jaren ook steeds meer op industrieel niveau toegepast om het aantal stappen in een uitgebreide synthese te verminderen en zo het proces schoner te maken. De afgelopen vijftien jaar is er veel onderzoek gedaan naar het gebruik van enzymen voor de synthese van polymeren. Verscheidene enzymen en monomeren zijn getest, maar accurate informatie over het werkelijke proces is tot nu toe nauwelijks verkregen. Bovendien heeft bestaand onderzoek zich voornamelijk gericht op de synthese van al bestaande materialen, welke ook met behulp van een chemische katalysator gemaakt kunnen worden.

In dit promotie-onderzoek wordt de toepassing van enzymen in de polymeersynthese nader onderzocht. Daarbij staat de volgende vraag centraal: "Wat kunnen enzymen toevoegen aan de polymeerchemie?" Novozym 435™, een commercieel verkrijgbaar, geïmmobiliseerde vorm van *Candida antarctica* Lipase B, is gebruikt als enzym vanwege de hoge activiteit, de thermostabiliteit en het gemak in het gebruik. Verder is het onderzoek toegespitst op de ring-opening polymerisatie van lactonen. Het doel van dit onderzoek is om meer accurate informatie te verkrijgen over het enzymatische polymerisatie proces. Daarnaast is er gekeken of dat met behulp van enzymen nieuwe materialen gemaakt kunnen worden die niet met behulp van chemische katalysatoren toegankelijk zijn.

ε-Caprolacton (ε-CL), een cyclisch ester, is gekozen als monomeer in de enzymatische ring-opening polymerisatie, aangezien dit monomeer het meest beschreven is in de literatuur. Door reactiecondities zoals de temperatuur, de concentratie monomeer, de soort initiator en de hoeveelheid water in het systeem te optimaliseren konden goed gedefinieerde polymeren gemaakt worden. Alleen door het optimaliseren van deze condities kon de vorming van bijproducten, zoals polymeerketens met andere eindgroepen, voorkomen worden. Om een inschatting te kunnen maken van de mate van effectiviteit van de enzymatische polymerisatie, werd een analysetechniek uitgewerkt die de polymeerketens met verschillende eindgroepen kon scheiden en kwantificeren, namelijk vloeistof chromatografie bij kritische omstandigheden (LCCC). De resultaten verschaften een beeld van welke ketens op welk moment gedurende de polymerisatie het meest gevormd werden. Water, en niet de initiator,

blijkt de voornaamste nucleofiele component te zijn aan het begin van de polymerisatie, zelfs wanneer de water concentratie erg laag is. Door nucleofiele competitie met het aanwezige water wordt de initiator langzamer ingebouwd gedurende de polymerisatie, afhankelijk van de activiteit van de initiator. Gedurende de hele reactie worden cyclische polymeerketens gevormd en de hoeveelheid hiervan hangt af van de initiële monomeer concentratie.

Deze nieuwe kennis werd gebruikt om de compatibiliteit van enzymen te onderzoeken in een chemisch gekatalyseerde polymerisatie (atom transfer radical polymerisation, ATRP). Met behulp van een bifunctionele initiator en de twee intrinsiek verschillende polymerisatietechnieken werden blokcopolymeren gesynthetiseerd. Alle bijproducten in de enzymatische ring-opening polymerisatie zouden daarbij resulteren in een vermindering van de opbrengst van het blokcopolymeer. De blokcopolymeren zijn via verschillende synthese routes gemaakt, waarbij werd gevonden dat het enzym wordt gedeactiveerd in aanwezigheid van de chemische katalysator (een overgangsmetaal). Hieruit blijkt dat een directe synthese van het blokcopolymeer zonder de vorming van bijproducten alleen mogelijk wanneer de enzymatische polymerisatie voor de ATRP-reactie wordt uitgevoerd.

Om nieuwe materialen te maken met enzymen werd een groter lacton gekozen, (ω-pentadecalactone, PDL). Door het gebrek aan ringspanning in macroliden (zoals PDL) kunnen deze monomeren niet goed met behulp van chemische katalysatoren worden gepolymeriseerd tot een hoog molecuulgewicht. Enzymen echter blijken de polymerisatie van deze monomeren zeer efficiënt te katalyseren. Door de 'schone' synthese van dit type monomeren wordt een nieuw type materialen beschikbaar, dat onder andere voor biomedische toepassingen gebruikt kan worden. De enzymatische ring-opening polymerisatie van PDL is opgeschaald zodat 30 gram polymeer per batch werd verkregen, dat vervolgens gebruikt kon worden voor het testen van de mechanische eigenschappen. Het doel daarbij was om een zo hoog mogelijk molecuulgewicht te bereiken met een smalle molecuulgewichtsverdeling, wat het polymeer de ideale eigenschappen zou geven voor vezeltoepassingen. Van de verkregen polymeren zijn vezels gemaakt met behulp van een smeltspin-proces en de mechanische eigenschappen (kracht-rek curve) van de PPDL-vezels bevestigen de potentie van dit nieuwe materiaal.

Dit proefschrift heeft aangetoond dat enzymen een aanzienlijk potentieel hebben binnen de polymeerchemie. Gedetailleerd onderzoek naar de enzymatische ring-opening polymerisatie van ε-CL heeft de kritische parameters voor dit proces aan het licht gebracht. Enzymatische reacties kunnen compatibel zijn met chemische polymerisatietechnieken, waardoor nieuwe polymere structuren beschikbaar zijn geworden. Tenslotte hebben enzymen de potentie om nieuwe polymeren te maken, die niet met behulp van chemische katalysatoren synthetiseerbaar zijn. Dit opent geheel nieuwe perspectieven binnen dit toepassingsgebied.

Curriculum Vitae

Matthijs de Geus werd geboren op 17 december 1978 in Groningen. In 1996 behaalde hij zijn gymnasium diploma aan het Mencia de Mendoza Lyceum te Breda. In datzelfde jaar begon hij met de opleiding Scheikundige Technologie aan de Technische Universiteit Eindhoven. In augustus 2002 legde hij met succes het doctoraalexamen van deze opleiding af, waarna hij tot eind december een tijdelijk onderzoekscontract had bij dr. ir. L. Klumperman in dezelfde onderzoeksgroep. Per 1 januari 2003 startte hij zijn werkzaamheden als promovendus aan de Technische Universiteit Eindhoven onder begeleiding van prof. dr. C.E. Koning en dr. A. Heise.

Matthijs de Geus was born in Groningen on December 17th 1978. In 1996, he finished gymnasium at Mencia de Mendoza Lyceum in Breda. Subsequently, he started his Master studies in Chemical Technology at the Technische Universiteit Eindhoven. After graduation in August 2002 with a major in Polymer Chemistry, he worked temporarily as a researcher for dr. ir. L. Klumperman in the same research group. From January 2003 he was employed as a PhD-student under guidance of prof. dr. C.E. Koning and dr. A. Heise at the Technische Universiteit Eindhoven.

List of publications

"*End-group fidelity in enzymatic polymerizations*"; M. de Geus, R. Peters, C.E. Koning, A. Heise, *to be submitted.*

M. de Geus, E. Custers, C.E. Koning, A. Heise, *in preparation.*

M. de Geus, R. Peters, C.E. Koning, A. Heise, *in preparation.*

M. de Geus, R. Peters, Y. Mengerink, C.E. Koning, A. Heise, *in preparation.*

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"Enzymatic catalysis in the synthesis of poly(lactones-co-methacrylate) block copolymers"; M. de Geus, A.R.A. Palmans, C.E. Koning, A. Heise, *Polymer Preprints*, **2003**, *44*(2), 613-614.

Dankwoord

Nu ik op het punt sta om de laatste hand te leggen aan dit proefschrift en daarmee aan vier jaar hard zwoegen, herinner ik mij een artikel uit de Chicago Tribune*. Dit artikel lijkt mij op het lijf geschreven, hoewel ik dat op dat moment nog niet helemaal door had...

'Climbing the steps to the final degree is a difficult trek'

'The dissertation journey has parallels to hiking up a mountain. Both involve unusual and sometimes extreme discipline and devotion. For success, both require careful planning and favorable travel conditions. These conditions involve a solid financial base, a solid knowledge base, and a solid emotional base. Not only are you ready but you are an expert in your own right. Your committee (assuming it's benign) knows this and wants you to succeed. Like a guide, they've climbed a mountain and do want to help you.'

Om te beginnen wil ik mijn promotor Cor Koning hartelijk bedanken, die mij de mogelijkheid geboden heeft om dit project uit te voeren. Cor, je positieve blik op het onderzoek en het vertrouwen dat je me schonk zal ik niet vergeten. Voorts wil ik de twee copromotoren bedanken, Andreas Heise en Anja Palmans, die mij tijdens deze vier jaren met raad en daad hebben bijgestaan en gestimuleerd. Het was een voorrecht om met jullie te mogen samen werken. Jan Meuldijk dank ik hartelijk voor de betrokkenheid die hij toonde, vooral bij het schrijfproces. Ook al werd je pas laat bij dit onderzoek betrokken, je adviezen waren zeer welkom. De overige leden van de kerncommisie dank ik hartelijk voor het kritisch doorlezen van mijn proefschrift. Verder zou ik graag Ron Peters en Harm van der Werff bij DSM willen bedanken voor de inspanningen die ze geleverd hebben om dit onderzoek tot een succes te maken. Verder wil ik DPI bedanken voor de financiële ondersteuning van dit project.

'This thing is to persist. And persist. And continue persisting... The mountain that is the dissertation work is a tremendous challenge. At times the university, the data/population, your computer can be obstacles as well. If it were easy everyone would summit mountains and be called "Doctor"'.
'Remember, it is OK to ask for help. In fact, with mountains and big papers we all need support. From alpine guides, faculty members and seasoned secretaries to friends and family, everybody needs direction and warm words. Doing a dissertation can be lonely and we all can use a little community.'

* Chicago Tribune (09/21/03) – '*The dissertation as Xtreme sport*' by Dr. Christopher Gallup

Daarom wil ik een ieder die mij de afgelopen vier jaar heeft bijgestaan in het onderzoek bedanken. Bart van As, Joris Peeters, Lars van der Mee en Thomas Hermans en Martin Wolffs, bedankt voor jullie nuttige suggesties, commentaar en diverse resultaten die mij het onderzoek vereenvoudigden. Erica Custers, Linda Schormans en Harro Antheunis, ik hoop dat jullie je bijdrage in dit proefschrift hebben herkend. I also would like to thank the members of the enzyme-family in the SPC-group, David, Jenny and Gaetan, for the discussions we had about this wonderful topic.

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> *'Finally, we are all going to get bruised along the way. There's so much energy and time involved in going from proposal to defence. So pursuing this path of a thousand steps/tasks involves sacrifices and scars. Each day's walk helps you push past the pains toward the vision of the final chapter. The summit becomes reachable.*

Nu de top in het zicht gekomen is, kom ik aan bij de mensen die het dichtst bij me staan. Ik wil graag de mensen bedanken die mijn thuisfront vormen. Bedankt voor alle steun en support, ook al kreeg ik (sommigen) maar niet uitgelegd wat ik nou precies deed. Jullie aandacht en zorg hebben mij de gehele periode een warm gevoel gegeven.

> *'Finally as you defend, and when you receive your degree, you'll feel pride, satisfaction, completion and a belief that it was all worthwhile. You've been there, done it and now can wear the T-Shirt.'*

De allerlaatste is voor de allerliefste: Anneloes, bedankt!

'The long climb is over, a new journey begins.'

Matthijs