Lipase Specificity and Selectivity
Engineering, Kinetics and Applied Catalysis

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Doctoral Thesis

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Cover picture:
*Candida antarctica* lipase B:
The catalytic triad amino acids (Ser105, Asp187 and His224) are shown in ball-and-stick.
Trp104 and Gln157 are shown in space fill.

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Abstract

The specificity and selectivity of the enzyme *Candida antarctica* lipase B (CALB) were studied for several substrates and applications. With help of molecular modeling, the active site of CALB was redesigned for the ring opening polymerization of $\textit{D,D}$-lactide. Two mutants, with about 90-fold increase in activity as compared to the wild-type enzyme, were created. Changing a glutamine into alanine accounted for this increase in both mutants by creating a larger space in the acyl donor pocket. The new space made it possible to accommodate the bulky substrate and improve the transition state-active site complementarity during polymer chain propagation.

The enantioselectivity of CALB towards secondary alcohols was engineered by rational redesign of the stereoselectivity pocket in the enzyme active site. A larger space created by a single point mutation resulted in an $8'300'000$ times change in enantioselectivity towards 1-phenylethanol and the enantiopreference was inverted into $S$-preference. The activity towards the $S$-enantiomer increased $64'000$ times in the mutant as related to the wild-type. The solvent and temperature effects on the enantioselectivity were studied for several substrates and revealed the importance of entropy in the change in enantioselectivity.

Substrate selectivity is of great importance for the outcome of enzyme catalyzed polymer synthesis. Ring opening polymerization (ROP) of $\gamma$-acyloxy-$\epsilon$-caprolactones will result in a polyester chain with pendant functional groups. CALB was found to have activity not only towards the lactone but also towards the $\gamma$-ester leading to rearrangement of the monomers yielding $\gamma$-acyloxyethyl-$\gamma$-butyrolactone. This selectivity between the lactone and the $\gamma$-ester was dependent on the type of group in the $\gamma$ position and determined the ratio of polymerization and rearrangement of the monomers. Molecular dynamics simulations were used to gain molecular understanding of the selectivity between the lactone and $\gamma$-ester.

In order to obtain (meth)acrylate functional polyesters we investigated the use of 2-hydroxyethyl (meth)acrylate (HEA and HEMA) as initiators for ring opening polymerization. We found that, in addition to the ring opening polymerization activity, CALB catalyzed the transacylation of the acid moiety of the initiators. The selectivity of CALB towards the different acyl donors in the reaction resulted in a mixture of polymers with different end groups. A kinetic investigation of the reaction showed the product distribution with time when using HEA or HEMA with $\epsilon$-caprolactone or $\omega$-pentadecalactone.

The high selectivity of CALB towards lactones over (meth)acrylate esters such as ethylene glycol di(meth)acrylate was used to design a single-step route for the synthesis of di(meth)acrylated polymers. By mixing $\omega$-pentadecalactone with the ethylene glycol di(meth)acrylate and the enzyme in solvent free conditions, we obtained >95 % of di(meth)acrylated polypentadecalactone.

Taking advantage of the high chemoselectivity of CALB, it was possible to synthesize polyesters with thiol and/or acrylate functional ends. When using a thioalcohol as initiator CALB showed high selectivity towards the alcohol group over the thiol group as acyl acceptor for the ROP reaction. The enzymatic ability of catalyzing simultaneous reactions (ROP and transacylation) it was possible to develop a single-step route for the synthesis of difunctionalized polyesters with two thiol ends or one thiol and one acrylate end by mixing the initiator, lactone and a terminator.
List of publications

Mohamad Takwa, Marianne Wittrup Larsen, Karl Hult, Mats Martinelle. 
Manuscript.

Cristian Vaida, Mohamad Takwa, Mats Martinelle, Karl Hult, Helmut Keul, Martin Möller. 

III. Lipase Catalyzed HEMA Initiated Ring-Opening Polymerization: In Situ Formation of Mixed Polyester Methacrylates by Transesterification. 
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V. An S-Selective Lipase Was Created by Rational Redesign and the Enantioselectivity Increased with Temperature. 
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VI. Single-Step, Solvent Free Enzymatic Route to α,ω-Functionalized Polypentadecalactone Macromonomers. 
Mohamad Takwa, Karl Hult, Mats Martinelle. 

VII. One-Pot Difunctionalization of Poly-(ω-pentadecalactone) with Thiol-Thiol or Thiol-Acrylate Groups, Catalyzed by Candida antarctica Lipase B. 
Mohamad Takwa, Neil Simpson, Eva Malmström, Karl Hult, Mats Martinelle. 

* Shared first authorship
Additional papers

Thiol-Functionalized Poly(ω-pentadecalactone) Telechelics for Semicrystalline Polymer Networks.

Di-acrylate or Thiol-acrylate Functionalised Poly(ω-pentadecalactone) for Single Component, Semi-crystalline Photo-curable Thermoset Films.
Table of contents

1. Introduction ......................................................................................1
   1.1 Biocatalysis .................................................................................1
   1.2 Enzyme specificity .......................................................................1
   1.3 Enzyme selectivity ......................................................................3
   1.4 Enzyme engineering ...................................................................4
      - Rational redesign ......................................................................4
      - Random mutagenesis ............................................................5
   1.5 Synthetic biodegradable polymers ..........................................5
      - Enzymes in polymer synthesis ..............................................6
      - Enzyme-catalyzed polyester synthesis ...................................6
      - Functional polymers ............................................................8
   1.6 Candida antarctica lipase B ....................................................9

2. Substrate specificity........................................................................11
   2.1 Redesigning the specificity towards the polymerization of lactide ....11

3. Substrate selectivity........................................................................15
   3.1 Ring opening vs. rearrangement of monomer ............................15
   3.2 Substrate selectivity causes the formation of mixed polyester acrylates ....20
   3.3 Substrate selectivity as tool to make polyester (meth)acrylates ..........25

4. Enantioselectivity..........................................................................28
   4.1 Redesigning the enantioselectivity towards secondary alcohols ..........28

5. Chemoselectivity..........................................................................34
   5.1 Enzymatic synthesis of end-functionalized polyesters ...............34
      - Enzymatic synthesis of polymers with two thiol groups ............35
      - Enzymatic synthesis of polymers with thiol and acrylate end groups ....37

6. Conclusions ...............................................................................40

7. Acknowledgments ........................................................................42

8. References .................................................................................44
1. Introduction

1.1 Biocatalysis

Enzymes are biocatalysts that are necessary in all living systems to catalyze all chemical reactions required for their survival and reproduction. Enzymes are proteins with exceptions of ribozymes, which are RNA molecules with a catalytic activity. One of the important features of enzymes is their high catalytic efficiency sometimes at or near the diffusion limit\(^1\) with the ability of catalyzing reactions with up to \(10^{19}\)-fold rate acceleration.\(^2\) Enzymes can act efficiently as catalysts outside living systems (\textit{in vitro}), exhibiting a great condition promiscuity by catalyzing reactions under different conditions than their natural ones such as high temperature, reduced pressure, extreme pH, presence of organic solvents and in pure liquid or melted substrates. Many enzymes accept substrates that are structurally distinct from what they accept in their natural environment. In addition to this, enzymes have other properties such as specificity and selectivity. These excellent features of enzymes make them an attractive tool in organic and polymer chemistry,\(^3\,4\) detergents,\(^5\) agrochemical\(^6\) and food industries\(^7\) and for the production of fine chemicals\(^8\) and pharmaceuticals.\(^9\)

1.2 Enzyme specificity

Depending on their role in the organism, enzymes have specificity towards the reaction they catalyze and the substrate they use.

\textit{Reaction specificity}

The enzyme-substrate binding occurs in a region (active site) of the enzyme that contains one or a few amino acids responsible for the catalysis which are called catalytic amino acids. The type of the catalytic amino acids depends on the type of reaction that the enzyme catalyzes. This gives the enzyme specificity for the reaction it catalyzes. The importance of the reaction specificity could be shown by the following example: three different enzymes,
transaminase (transferase, EC 2.6.), decarboxylase (lyase, EC 4.1.) and dehydrogenase (oxidoreductase, EC 1.1.) use the same substrate, glutamic acid, giving three different reaction products. The transaminase will give α-ketoglutaric acid and a new amino acid. With the decarboxylase the corresponding amine and carbon dioxide will be produced. The dehydrogenase will give α-ketoglutaric acid and ammonia (Figure 1). This specificity is of a great importance in the living organism because if one enzyme could catalyze several reactions, there will be no control of the amount and type of product. There are some exceptions where an enzyme shows the ability of catalyzing several reactions in the same active site (catalytic promiscuity). The enzyme Rubisco is a good example of natural catalytic promiscuity where in addition to the CO₂ fixation in the photosynthesis, it could catalyze the fixation O₂ resulting in a by-product that needs a different metabolic pathway.¹⁰

![Chemical structures](image)

**Figure 1:** An example of reaction specificity. One substrate gives three different products depending on the enzyme that catalyzes the reaction.

**Substrate specificity**

Substrate specificity describes how good an enzyme is at catalyzing the conversion of a substrate. The identity and arrangement of the amino acids in the active site define the specificity towards the substrates. Structural complementarity between the transition state and the active site together with the electrostatic interactions and the hydrophobicity of the active site are the properties that determine the specificity. The substrate specificity is
determined by the specificity constant \((k_{cat}/K_M)\) which describes the catalytic efficiency of the enzyme towards a given substrate. In biocatalysis, engineering of the substrate specificity is of great importance in order to improve the efficiency of processes. In Paper I, an improved substrate specificity was successfully achieved by enzyme engineering.

1.3 Enzyme selectivity

Substrate selectivity

An important characteristic of enzymes is selectivity, that is their ability to discriminate between two different substrates. The selectivity value, between two substrates A and B, is expressed by the ratio of specificity constants of the two substrates \((k_{cat}/K_M)_A/(k_{cat}/K_M)_B\).

There are different types of substrate selectivity such as:

- **Chemoselectivity:** Enzymes can discriminate between substrates having different chemical groups. For example, *Candida antarctica* lipase B (CALB) shows \(10^5\) times higher selectivity for alcohols than for thiols in transacylation reactions.\(^{11}\)

\[
\begin{align*}
\text{HS} & \quad \text{CALB} \\
\text{R} & \quad \text{n} \\
\text{O} & \quad \text{O} \\
\text{O} & \quad \text{R}_1 \\
\end{align*}
\]

- **Regioselectivity:** Enzymes can be selective for one of two similar groups on the same substrate molecule. Hexokinase catalyzes the phosphorylation of glucose and exclusively produces glucose-6-phosphate, but no other glucose phosphate (i.e. glucose-1-phosphate or glucose-3-phosphate) is produced during the reaction.\(^{12}\)

\[
\begin{align*}
\text{HO} & \quad \text{OH} \\
\text{HO} & \quad \text{OH} \\
\text{HO} & \quad \text{OH} \\
\text{HO} & \quad \text{OH} \\
\text{OH} & \quad \text{OH} \\
\text{HO} & \quad \text{OH} \\
\text{HO} & \quad \text{OH} \\
\end{align*}
\]
- **Stereoselectivity**: Enzymes show selectivity between stereoisomers of a chiral substrate molecule. This means that one of the isomers reacts faster than the others. A good example is the enzyme D-amino acid oxidase that shows a high enantioselectivity for the D-amino acids and has no activity towards the oxidation of L-amino acids.\(^\text{13}\)

\[
\begin{align*}
\text{R} & \text{COOH} \quad \text{FAD} \quad \text{FADH}_2 \quad \text{NH}_2 \quad \text{H}_2\text{O} \quad \text{NH}_3 \quad \text{R} \text{COOH} \\
\text{D-amino acid oxidase} & \quad \text{H} \quad \text{O} \quad \text{H}_2\text{O} \\
\text{R} & \text{COOH} \quad \text{L} \\
\end{align*}
\]

1.4 Enzyme engineering

Engineering an enzyme means introducing changes in the amino acid sequence of the enzyme aiming at an improved catalyst for a given reaction or process. An increasing number of engineered enzymes are produced and used in various applications.\(^\text{14}\) Two main methods have been extensively used for engineering of enzymes; rational redesign and random mutagenesis.

**Rational redesign**

Rational redesign is a method of enzyme engineering based on structural information of the enzyme obtained by crystallography, NMR or from homology modeling. In this strategy one or more amino acids, believed to be important for the property that needs to be improved, will be targeted for site-directed mutagenesis. No screening method is needed when using this method due to the relatively small number of mutations that are usually introduced. Many enzymes have been rationally redesigned for different purposes such as (i) increasing the thermostability by changing amino acid in a loop replacing a glycine with a proline\(^\text{15}\) or changing amino acids in the high B-factor regions\(^\text{16}\) (ii) increasing or inverting the enatioselectivity (**Paper V**) (iii) improving the substrate specificity (**Paper I**) (iv) introducing catalytic promiscuity by introducing a new reaction specificity in an enzyme.\(^\text{17}\) Molecular modeling is a method that could be of great help in rational redesign. Molecular dynamics
simulations give a molecular understanding of the enzyme-substrate interactions in the transition state. In Paper I, II and V, molecular modeling studies were performed in which the substrates were modeled as tetrahedral intermediates in the active site of the enzyme. A kinetic investigation is usually of importance to obtain detailed molecular information about the engineered enzyme. In Paper I the rates of ring opening polymerization reaction (ROP) of CALB and the mutants were analyzed and compared. In Paper V the kinetic parameters ($k_{cat}$ and $K_M$) and the thermodynamic components were studied and compared for transacylation reactions of both enantiomers of a secondary alcohol.

**Random mutagenesis**

The gene of an enzyme can be subjected to random mutagenesis using one of the mutagenic methods such as error-prone PCR, gene shuffling or chemical mutagenesis. These methods do not require any knowledge about the enzyme structure, active site or reaction mechanism but on the other hand a screening method is needed to find the desired property. After obtaining a catalyst with improved property a second cycle of mutagenesis could be performed to further improve the catalyst.

**1.5 Synthetic biodegradable polymers**

Synthetic biodegradable polymers are a class of polymers designed to decompose after their functional purpose is over. They might be an environmentally friendly alternative to conventional polymers such as polyethylene and polypropylene. Biodegradable polymers are a good option for applications that require short term usage before disposal. In addition to the ability of biodegradation into harmless constituents, biodegradable polymers could offer functional properties similar to that of conventional polymers such as polyethylene. In a disposal environment, biodegradable polymers start to degrade either by enzymatic actions of microorganisms (bacteria, fungi and algae) or by non-enzymatic actions (chemical actions). Biodegradation converts the polymer chains into CO$_2$, CH$_4$, H$_2$O and other basic constituents. Target markets for biodegradable polymers include packaging materials, hygiene products, consumer goods, agricultural tools and biomedical applications.
Some of the common synthetic biodegradable polymers are polycaprolactone (Paper II, III and IV), polylactides (Paper I), polyhydroxybutyrate and polybutylene succinate.

**Enzymes in polymer synthesis**

For the last two decades, the use of enzymes in polymerization reactions has been extensively studied. Enzyme catalysis has provided new strategies for the synthesis of different types of polymers. Polysaccharides, polyphenols, polyanilines, vinyl polymers, polyesters, polycarbonates, poly(amo{}no{}n{}i{}c{} acid)s, polyamines, DNA, polythioesters and proteins have been enzymatically synthesized with good efficiency. Three of the six classes of enzymes have been employed successfully in polymer synthesis. Polymers produced by respective enzyme family are given in Table 1.

**Table 1.** Classification of enzymes and in vitro production of typical polymers catalyzed by respective enzymes family.36

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Typical polymers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidoreductases</td>
<td>Polyphe{}nones, polyani{}lines, vinyl polymers, polyamine{}s</td>
</tr>
<tr>
<td>Transferases</td>
<td>Polysaccharides, polyesters, proteins, DNA</td>
</tr>
<tr>
<td>Hydrolases</td>
<td>Polysaccharides, polyesters, polycarbonates, poly(amine{}acid)s, polythioesters</td>
</tr>
<tr>
<td>Lyases</td>
<td>-</td>
</tr>
<tr>
<td>Isomerases</td>
<td>-</td>
</tr>
<tr>
<td>Ligases</td>
<td>-</td>
</tr>
</tbody>
</table>

**Enzyme-catalyzed polyester synthesis**

Aliphatic polyesters have been widely investigated due to their synthetic feasibility, availability at sufficiently high molecular weights for use as bulk materials and their ability to undergo hydrolytic and biological degradation. Hydrolases, and in particular lipases have been very successful in polyester synthesis via two different procedures: Polycondensation or ring opening polymerization (ROP). Hydrolases can catalyze the polycondensation of hydroxy acids (AB-type monomers) and the polycondensation of diacids or anhydrides with diols (AA, BB-type monomers). Lipases catalyze the ROP of cyclic esters, cyclic diesters (glycolides and lactids) and cyclic carbonates. The enzymatic catalysis of the polycondensation and ROP reactions shows many advantages over the conventional chemical catalysis. By using the enzymatic polycondensation process
strong acidic catalysts or high temperature are not required as in the chemical process.\textsuperscript{43} An acidic catalyst may cause a discoloration of the polymer and a high temperature might lead to dehydration of diols affecting the stoichiometry of the reaction.\textsuperscript{29} Enzymatic ring opening polymerization (eROP) is a good alternative to the use of organometallic catalysts, which are difficult to remove entirely from the produced polymers. These organometallic compounds are often toxic and thereby limiting the use of the produced polymers in biomedical applications. The first report of an enzymatic ring opening polymerization reaction appeared in 1993,\textsuperscript{40,44} where $\varepsilon$-caprolactone (7-membered ring) was polymerized using a lipase. Successively, the ring opening polymerization (ROP) of various lactones with small to large ring sizes (4- to 17-membered rings), were efficiently catalyzed by different lipases.\textsuperscript{45} Contrary to the chemical catalysis,\textsuperscript{46} large lactones (i.e. pentadecalactone (PDL)), were polymerized with a high efficiency by enzymes.\textsuperscript{18} In addition, chiral polyesters were obtained by enzymatic ring opening polymerization of racemic substituted lactones.\textsuperscript{47} Furthermore, random copolymers were enzymatically obtained by copolymerization of different lactones\textsuperscript{48} and lactones with lactide.\textsuperscript{49} Lipase-catalyzed ring opening polymerization has been used to graft polyesters onto a polystyrene based backbone.\textsuperscript{50} An enzymatic ring opening polymerization reaction can be divided into two steps: \textit{initiation} where a nucleophile is needed for the ring opening of the cyclic ester monomer (lactide or lactone), and \textit{propagation} where the hydroxyl end group of the opened lactide or lactone acts as a nucleophile for the ring opening of a new monomer unit (Scheme 1).
Functional polymers

Functional polymers are polymers that contain free functional groups, such as hydroxyls, amines, thiols, epoxids and (meth)acrylates. The presence of free functional groups in the polymer will facilitate further the improvement (depending on the type of functional group) of the physical and chemical properties, including viscosity, solubility, hydrophilicity, adhesion, and will also provide the ability to make more complex structures for new materials. The first pioneering work on the synthesis of functional polymers and their conversion to the final products appeared in 1947. There are two types of functional polymers depending on the position of the functional groups: pendant-functionalized polymers with functional groups as side groups on the polymer chain (Paper II) and end-functionalized polymers where functional groups are present as end groups of the polymer chain (Paper III, IV, VI and VII).

Taking advantage of the enzymatic selectivity, different types of functionalities could be introduced. Regioselective initiation has been reported by Cordova et al. where the initiation from only one of several hydroxyl groups of a glucoside was observed. Enatioselective initiation has been done using 1-phenylethanol. Due to the high enantioselectivity of the enzyme (Paper V) only the R-enantiomer was used by the enzyme for the initiation. In addition, chemoselective initiation using thioalcohols as initiators has been reported by Hedfors et al. and in Paper VI and VII. Because of the chemoselective
initiation of eROP from the alcohol group of the initiator, polyesters with free thiol ends were obtained. Chemical methods for functionalization of lactones with a thiol end often requires protection and deprotection steps.\textsuperscript{55}

Enzymes can have some limitations when using an initiator with a cleavable ester bond. In \textbf{Paper III} and IV we used 2-hydroxyethyl acrylate (HEA) and 2-hydroxyethyl methacrylate (HEMA) as initiators for eROP of lactones aiming at (meth)acrylate functional polyesters. However the lipase showed to have specificity towards the ester group of both HEA and HEMA resulting in a mixture of polymer products. A detailed kinetic study of the eROP reaction using HEA or HEMA as initiators gave a better understanding of the enzyme specificities towards the different moieties in the reaction.

\textbf{1.6 Candida antarctica lipase B}

The yeast \textit{Candida antarctica} produces two different lipases, A and B.\textsuperscript{56} The two lipases are very different; lipase A is more thermostabile, more active towards large triglycerides while lipase B is much more active towards the hydrolysis of a broad range of esters.\textsuperscript{57} \textit{Candida antarctica} lipase B (CALB) is a globular protein that belongs to the $\alpha/\beta$ hydrolase fold family.\textsuperscript{58} The polypeptide chain is composed of 317 amino acids and its molecular weight is about 33 kDa. The three dimensional structure was determined by X-ray crystallography in 1994.\textsuperscript{59} CALB has been successfully engineered for different applications such as change of enantioselectivity (\textbf{Paper V}), introduction of catalytic promiscuity,\textsuperscript{17} increase of substrate specificity (\textbf{Paper I}), and increase of thermal stability.\textsuperscript{60} The active site of CALB is situated in the core of the protein and the binding site has a funnel-like shape and highly hydrophobic amino acid residues envelop the cavity inner walls. CALB has, compared to other lipases, a very limited available space in the active site pocket which explains its high selectivity. This enzyme has the same reaction mechanism as serine proteases with a catalytic triad (Ser105, His224 and Asp187) and an oxyanion hole (Thr40 and Gln106). The reaction mechanism is illustrated in Scheme 2, which shows a transacylation reaction. A carboxylic ester (substrate 1) binds in the active site and the carbonyl carbon of the ester is then attacked by the catalytic Ser105 (nucleophile), going through the first transition state (TS1). This attack is promoted by His224, which acts as a general base and accepts a proton from Ser105.
During the attack the carbonyl bond becomes a single bond and the carbonyl oxygen becomes an oxyanion and forms three hydrogen bonds with the oxyanion hole (two to Thr40 and one to Gln106). The alcohol (product 1) leaves the active site and an acyl enzyme is formed. A nucleophile (substrate 2) performs a new nucleophilic attack on the carbonyl carbon of the acyl enzyme going through a second transition state (TS2) forming the transacylation product (product 2), which is released and the free enzyme is regenerated.

Scheme 2. Reaction mechanism of Candida antartica lipase B. The first substrate (ester group) enters the active site of the enzyme and goes through the first transition state (TS1) forming an acyl enzyme and the first product. A nucleophile then enters the active site attacking the acyl enzyme and goes through the second transition state (TS2) forming the free enzyme and giving the second product. The amino acids forming the oxyanion hole are only shown for the transition states.
2. Substrate specificity

Many enzymes show the ability to catalyze a wide range of reactions at different ranges of efficiencies depending on the structural complementarity of the transition state with the active site. In Paper I the substrate specificity of CALB for ring opening polymerization of lactides has been engineered by rational redesign improving the complementarity between the transition state and the active site.

2.1 Redesigning the specificity towards the polymerization of lactide

CALB shows different activities towards ROP of the two stereoisomers of lactides (\(D,D-(R,R)\) and \(L,L-(S,S)\) lactide). While no polymer formation was detected using the \(L,L\)-lactide, 33% conversion was achieved after 3 days when using the \(D,D\)-lactide at 70 °C in toluene.\(^6^1\) The eROP of \(D,D\)-lactide proceeds in two steps, initiation and propagation as described in the introduction (Scheme 1). The specificity towards the initiation step was not the reason of the low activity of the polymerization since Jeon et al. have shown that CALB has a good activity for the alcoholysis reaction of \(D,D\)-lactide.\(^6^2\) This indicates that the low activity of CALB towards the ROP of \(D,D\)-lactides occurs during the propagation step. In order to get a molecular understanding of the propagation step and to design an enzyme more active towards the \(D,D\)-lactide, a molecular modeling study was done. The propagation step was modeled by building the substrate as a tetrahedral intermediate in the enzyme, where a benzyl dilactate had made a nucleophilic attack on a dilactate-acylated enzyme (Figure 2).

By a molecular dynamics simulation, one amino acid in the acyl donor pocket (Gln157) and two amino acids around the entrance of the active site (Ile189 and Leu278) were suggested as targets for making a larger space in the active site to accommodate the bulky reactants (Figure 3). Molecular dynamics simulations that were run on the enzyme variant (Q157A, I189A, L278A) showed that the substrate gained a relaxed conformation in the active site in which the acyl acceptor was pointing towards the entrance of the active site and the acyl donor had a nicer fit in the new large space in the acyl donor pocket compared to the WT (Figure 3). The essential hydrogen bonds (two from His224 and two from the oxyanion hole)
that were absent in the wild type structure were found to be formed in the mutant enzyme after dynamics. The following mutants were constructed using site directed mutagenesis: (Q157A), (I189A), (L278A), (I189A, L278A) and (Q157A, I189A, L278A). Initial screening of the mutants and WT CALB together with *Humicola insolens* cutinase, towards the ring opening polymerization of \( D,D \)-lactide revealed that mutants (Q157A) and (Q157A, I189A, L278A) had the highest improvements in polymerization as compared to WT. These two mutants were chosen for a detailed kinetic study. In spite of its open active site, *Humicola insolens* cutinase showed a low activity towards the polymerization of \( D,D \)-lactides indicating that a large active site is not enough to guarantee a better transition-state active site complementarity. Mutant (Q157A) showed a 4-fold improvement in the initiation step while the triple mutant showed a 20-fold increase (Table 2). The space created around the entrance of the active site having alanines in positions 189 and 278 increased the initiation rate and a large acyl acceptor molecule such as 1-phenyl ethanol would benefit from this large space. Mutants (Q157A) and (Q157A, I189A, L278A) showed around a 100-fold increase in reaction rate in the propagation step as compared to the WT (Table 2). The space created in the acyl donor pocket by replacing glutamine with alanine had a large impact on the propagation step by improving the acyl transfer.
Figure 3. Structures of the active site of WT CALB (left) and (Q157A, I189A and L278A) mutant (right) with the tetrahedral intermediate representing the propagation step where a lactide unit is the acyl donor and benzyl dilactate is the acyl acceptor. The mutated positions are shown in space fill. The substrate in the WT (ball-and-stick, left) has a crowded conformation in the active site due to the lack of space while the substrate in the mutant active site (ball-and-stick, right) has a nice fit due to the space created by alanine mutations in positions 157, 189 and 278. The substrate and the side chains of the mutated amino acids are displayed without hydrogens (Paper I).

Table 2. Rates (s⁻¹) of the initiation and propagation steps of the ROP reaction of D,D-lactide, and for the hydrolysis of tributyrin and transacylation of ethyl octanoate catalyzed by WT-CALB and mutants (Paper I).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>D,D-lactide Initiation</th>
<th>D,D-lactide Propagation</th>
<th>Tributyrin Hydrolysis</th>
<th>Ethyl octanoate Transacylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>40</td>
<td>1</td>
<td>330</td>
<td>200</td>
</tr>
<tr>
<td>(Q157A)</td>
<td>180</td>
<td>93</td>
<td>10</td>
<td>92</td>
</tr>
<tr>
<td>(Q157A, I189A, L278A)</td>
<td>770</td>
<td>83</td>
<td>230</td>
<td>28</td>
</tr>
<tr>
<td>(L278A)</td>
<td>-</td>
<td>-</td>
<td>1400</td>
<td>-</td>
</tr>
</tbody>
</table>

| a 60 °C in toluene (determined by ¹H NMR). | b Hydrolytic activity towards tributyrin run at 25 °C. | c Transacylation of ethyl octanoate with 1-hexanol at 25 °C in cyclohexane (determined by GC). |

Because of the increased space created, the efficiency of an aliphatic acyl acceptor such 1-hexanol for the transacylation reaction of ethyl octanoate dropped by approximately 50 % with mutant (Q157A) and 86 % with the triple mutant as compared to the WT (Table 2). Furthermore, by having alanine in position 278, the hydrolysis rate towards tributyrin increased remarkably as compared to the WT. The (L278A) mutant showed a 4-fold higher activity than the WT (Table 2), which has also been observed by Liu et al.⁶³ The space created by replacing leucine 278 with alanine might be of importance for the entrance of bulky substrates to the active site.
Preparative ring opening polymerization reactions were run using the WT and mutants (Q157A) and (Q157A, I189A, L278A). As shown in Table 3, the highest conversion (89 % after 48 hours) was achieved with mutant (Q157A, I189A, L278A) and polymers with molecular weight ($M_n$) of 780 Da were produced corresponding to a degree of polymerization (DP) of 4.5 lactide unites (9 lactic acid unites). Mutant (Q157A) showed a conversion of 70 % and polymers with $M_n$ of 680 Da, DP of 4 (Table 3). The WT enzyme showed 11 % conversion and polymers with $M_n$ of 280 Da, DP of 1 (Table 3) which indicates that the WT enzyme has catalyzed the initiation step only. By MALDI-TOF spectrum the mass difference between the peaks was found to be 72 Da (corresponds to one lactic acid unit) instead of 144 that corresponds to a lactide unit. This shows that the mutants had a transacylation activity during the polymerization reaction similar to what was seen when using hydroxyl ethyl (meth)acrylate (HEA and HEMA) as initiators for eROP (Paper III and IV).

In conclusion, the WT CALB shows low activity towards the $D,D$-lactide even though it has a high activity towards lactones. The reason behind this is the bulky conformation (with one sp$_2$ centre and the two methyl groups on the α-positions) of the lactide when acylated. The space created in the acyl donor pocket of the Q157A mutant improved the complementarity to the lactide as acyl donor when having the propagating chain as acyl acceptor (propagation step) improving the specificity. On the other hand, this space had a negative impact on the specificity towards substrates such as ethyl octanoate that already have a good complementarity with the WT active site. The activation entropy might be the reason of the latter loss in specificity since the new space created in the active site gives a higher degree of freedom of the enzyme-octanoate complex.

Table 3. Synthesis of poly($D,D$-lactide) catalyzed by WT CALB and its variants (Paper I).$^a$

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$M_n$ (Da)</th>
<th>PDI$^b$</th>
<th>DP$^c$</th>
<th>Conv. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>220</td>
<td>280</td>
<td>1.1</td>
<td>1</td>
</tr>
<tr>
<td>(Q157A)</td>
<td>560</td>
<td>680</td>
<td>1.6</td>
<td>4</td>
</tr>
<tr>
<td>(Q157A, I189A, L278A)</td>
<td>740</td>
<td>780</td>
<td>1.6</td>
<td>4.5</td>
</tr>
</tbody>
</table>

$^a$ 48 hours in D$_2$-toluene at 60 °C using 1-phenylethanol as initiator. $^b$ Determined by SEC. $^c$ Determined by $^1$H NMR.
3. Substrate selectivity

When the substrate has more than one site that the enzyme can act on, the enzyme might have different specificities towards the different sites based on their structure geometry and their reactivity. The selectivity can be quantified as the ratio between the specificities towards the different sites. In Paper II, molecular dynamics simulations were used to understand the difference in specificity between the two ester groups on the γ-acyloxy-ε-caprolactone molecules. The enzyme showed different specificities towards the γ-acyloxy group depending on its substituent. Based on the position of the ester group (lactone or γ-acyloxy), the enzymatic reactivity will determine the ratio of possible products formed. In Paper III and IV, a kinetic study on ROP was done using initiator molecules containing an ester bond. The study showed that these initiators in addition to being acyl acceptors also could function as acyl donors resulting in a mix of products.

3.1 Ring opening vs. rearrangement of monomer

Pendant functional groups give polymers additional properties and the ability, depending on the type of the functional group, of making further reactions. Using γ-acyloxy-ε-caprolactones as monomers when subjected to ROP will result in a polyester (polycapro-lactone) with pendant ester groups in γ-position (Scheme 3). When using CALB as a catalyst for the ROP of four different γ-acyloxy-ε-caprolactones (γ-methacryloyloxy-, γ-benzoyloxy-, γ-acryloyloxy-, γ-acetyloxy), in addition to the polymerization a rearrangement of the monomers yielding γ-acetyloxyethyl-γ-butyrolactone was seen (Scheme 3). The ratio of the polymerization and rearrangement products were depending on the moiety presented in the γ-position. In order to gain information on the substrate recognition displayed by CALB towards the γ-ester and the lactone ester of γ-acyloxy-ε-caprolactones (1 A-D, Scheme 3), a molecular dynamics (MD) study was performed. The γ-acetyloxy-ε-caprolactone (1A) and the γ-benzoyloxy-ε-caprolactone (1B) were chosen as substrates, since (1A) resulted exclusively in the rearrangement product (2A) while (1B) resulted in the polymer only (3B) (Table 4).
Scheme 3. Rearrangement of γ-acyloxy-ε-caprolactones vs. ring opening polymerization (ROP) (Paper II).

Table 4. Ring opening polymerization of γ-acetoxy-ε-caprolactone (1A) and γ-benzoyloxy-ε-caprolactone (1B) using CALB.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>I:M</th>
<th>Conversiona</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Polymerization (%)</td>
</tr>
<tr>
<td>1 A</td>
<td>1:50</td>
<td>0</td>
</tr>
<tr>
<td>1 B</td>
<td>1:50</td>
<td>63</td>
</tr>
</tbody>
</table>

aRatio initiator to monomer (3-phenylpropanol was used as initiator). b The reactions were run in toluene at 70°C.

Each substrate was built in the enzyme as a tetrahedral intermediate in two different ways: 1) Ser105 was covalently connected to the carbonyl carbon of the lactone (initiation step of ROP reaction) (Figure 4 A); 2) Ser105 was covalently connected to the carbonyl carbon of the γ-ester carbonyl (resulting in rearrangement products) (Figure 4 B). The results from the molecular modeling showed that substrates 1A and 1B can be accommodated in the active-site of CALB as productive transition states for both the γ-ester and the lactone ester with the essential hydrogen bonds developed as illustrated in Figure 5.
The structures of the lactone ester transition state of both substrates (γ-acetyloxy and γ-benzoyloxy-ε-caprolactone) showed that the γ-esters were pointing towards the entrance of the active site (Figure 5 A). The structures of the γ-ester TS of both substrates (γ-acetyloxy and γ-benzoyloxy) showed clearly that the γ-ester groups had to be situated deep in the active site which became crowded especially for the γ-benzoyloxy group (Figure 5 B). Analysis of the root mean square (rms) deviation of the protein backbone during the simulations showed larger structural changes for the γ-benzoyloxy ester TS as compared with the other three TS structures (Figure 6). This indicates that the lipase needs to make larger structural rearrangements in order to accommodate the benzoyloxy group as compared to the acetyloxy group in the γ-ester TS. In the lactone ester TS the enzyme structures for both γ-acetyloxy and γ-benzoyloxy tetrahedral intermediates were similar throughout the simulation (Figure 5 A and Figure 6). The structural changes observed during the simulation indicate unfavourable interactions in the binding of the γ-benzoyloxy ester TS in the enzyme and offered an explanation for the different products achieved for the γ-benzoyloxy and the γ-acetyloxy substrates and showed the importance of complementarity between the transition state and the active site. γ-Acetyloxy-ε-caprolactone could not be polymerized because the enzyme had a much higher specificity for the γ-ester than for the lactone ester. On the other hand the γ-benzoyloxy-ε-caprolactone resulted in polymers only since the γ-benzoyloxy group is bulky and does not have a good fit in the active site to stabilize the transition state. The low polymerization percentage is probably due to the steric hindrance during the propagation step.
**Figure 5.** MD simulated transition-state (TS) structures of γ-acyloxy-ε-caprolactones in the formation of the acyl enzyme in CALB. TS structures (substrate connected to Ser105) colored by atom-type. Catalytic acid/base (His224) and oxyanion hole residues (Gln106, Thr40) in yellow. Binding-site residues in CALB colored in green (γ-benzoyloxy-ε-caprolactone TS structures) and in blue (γ-acetyloxy-ε-caprolactone TS structures). **A:** The lactone ester TS of γ-acetyloxy-ε-caprolactone (binding site residues in blue) and γ-benzoyloxy-ε-caprolactone (binding site residues in green). The methyl group in the γ-acetyloxy group is colored in purple. **B:** The γ-ester TS of γ-acetyloxy-ε-caprolactone (binding site residues in blue) and γ-benzoyloxy-ε-caprolactone (binding site residues in green). The methyl group in the γ-acetyloxy group is colored in purple. *(Paper II)*

**Figure 6.** The RMS distance from the starting structure of the enzyme backbone atoms in the MD-simulations as a function of time *(Paper II)*.
Table 5. Ring opening polymerization of γ-acryloyloxy-ε-caprolactone and γ-methacryloyloxy-ε-caprolactone using CALB.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>I:M</th>
<th>Conversion</th>
<th>Polymerization (%)</th>
<th>Rearrangement (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Molecule" /></td>
<td>1:30</td>
<td>20</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td><img src="image2.png" alt="Molecule" /></td>
<td>1:30</td>
<td>45</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

*Ratio initiator to monomer (3-phenylpropanol was used as initiator). The reactions were run in toluene at 25 °C.

From the experimental results we also observed an interesting difference in selectivity between γ-acryloyloxy- and γ-methacryloyloxy-ε-caprolactone. The difference in specificity is believed to be caused by having different groups in the γ-position and is resulting in a different ratio of polymers and rearrangement products. When using γ-acryloyloxy-ε-caprolactone 75% of the monomer resulted in rearrangement products and 20% in polymers while with γ-methacryloyloxy-ε-caprolactone 45% of the monomers resulted in polymers and no rearrangement was observed (Table 5). CALB has been shown to have higher specificity towards acrylate as compared to methacrylate due to the difference in chemical nature of both groups and the steric hindrances caused by the methyl group. This is the reason for the high percentage of rearrangement products when γ-acryloyloxy-ε-caprolactone is used as a substrate.
3.2 Substrate selectivity causes the formation of mixed polyester acrylates

The functionalization of macromonomers with vinyl groups such as (meth)acrylate or styrene, allows further synthesis of more complex polymer architectures using radical polymerization methods. These can be divided in two types; the classical radical growth method and the controlled radical polymerization method. Dubois et al. successfully synthesized mono- and di-methacrylated poly-caprolactone using a chemical catalyst.\(^{65,66}\) Shinoda et al. introduced chemically a methacrylate group to poly(lactic acid). The macromonomers were then copolymerized using controlled radical polymerization.\(^{67}\) No success has been achieved in introducing acrylate groups to polymer chains of large lactones non-enzymatically, but (meth)acrylate groups have been used enzymatically in the ring opening polymerization either as initiators in the form of 2-hydroxyethyl (meth)acrylate or as terminators as methyl, ethyl or vinyl acrylate. Previously, three research groups have used 2-hydroxyethyl methacrylate (HEMA) as initiator for the ring opening polymerization of \(\varepsilon\)-caprolactone and \(\omega\)-pentadecalactone resulting in the formation of HEMA initiated polymers.\(^{68,69,70}\)

In Paper III and IV, 2-hydroxyethyl acrylate (HEA) and HEMA were used as initiators for the ring opening polymerization of \(\omega\)-pentadecalactone and \(\varepsilon\)-caprolactone (Scheme 4 A). We found that the ester group of the initiators was itself a substrate for the enzyme, resulting in a mixture of products with various end groups. By \(^1\)H-NMR and MALDI-TOF MS, we found that the lipase did not only catalyze the HEA/HEMA initiated ROP but also the cleavage of the ester bond within the HEA/HEMA-moiety of the polymer. This cleavage resulted in two major types of acyl transfer reactions; (meth)acrylate transfer and polyester acyl transfer (Scheme 4 B and C). The (meth)acrylate transfer (Scheme 4 B) led to polymers with four different end group structures; HEA/HEMA end groups (1, 3), hydroxyl end groups (1, 2), 1,2-ethanediol end groups (2, 3) and (meth)acrylated hydroxyl end groups (3). Furthermore, as a result of the polyester acyl transfer reaction (Scheme 4 C), the 1,2-ethanediol moiety was found within the polyester chain (4). The presence of these structures was confirmed for both PCL and PPDL.
As stated earlier in the introduction, a covalent acyl enzyme intermediate (acyl enzyme), is formed by a nucleophilic attack on the carbonyl carbon of the acyl donor by the serine 105.

In Scheme 4, three different acyl enzymes are illustrated: (A) a lipase acylated by the opened monomer; (B) a lipase acylated by the (meth)acrylate moiety; (C) a lipase acylated by a polyester moiety. The formed acyl enzymes are subsequently deacylated: in (A) by the initiator (initiation) or the growing polyester (propagation); in (B) by the hydroxyl end group of the polymer; and in (C) by the ethanediol moiety of the polymer.
Figure 7. Kinetic studies of CALB catalyzed ROP of PDL (A) and CL (B) initiated with HEA or HEMA. Conversion of the lactone with time; HEA initiated (■); HEMA initiated (○). The amount of free initiator, HEA (●) and HEMA (○) (Paper IV).

We conducted a kinetic investigation on the CALB activity towards HEA and HEMA as acyl acceptors (initiators, see reaction A, Scheme 4) for the polymerization of PDL and CL and as acyl donors (acyl transfer, see reaction B, Scheme 4). The conversion of the lactones PDL and CL and the consumption of the initiator (HEA or HEMA) as acyl acceptor are plotted against time in Figure 7. CALB was found to catalyze ROP of PDL about 7-fold more efficiently as compared to CL. PDL was fully converted into polymer after 3 h in the presence of HEA or HEMA while the CL polymerization was slower. Michaelis-Menten kinetic parameters of the ring opening of various sized lactones, using two different lipases, show a higher specificity towards large lactones as compared to smaller ones.71,72,73 Kobayashi et al. attributed this difference in specificity to the increased hydrophobicity of the large lactones which better promote the formation of the lactone-enzyme complex.74 Palmans’s group has suggested that the difference in specificity is due to the conformation of the lactones, where large lactones with low ring strain assume mainly a favourable transoid conformation of the ester bond in contrast with small and medium sized lactones that have unfavourable cisoid conformations.73

CALB used HEA and HEMA with a similar efficiency in the eROP since a similar reaction rate profile was observed for both. The structural difference between HEA and HEMA (the additional methyl group in HEMA) did not result in any major steric interactions at the active site that would have lowered the initiation efficiency of the HEMA. The consumption of HEA and HEMA followed a similar pattern with PDL (Figure 7 A) and also with CL (Figure 7 B).
In the case of CL, the conversion of monomer was slightly faster with HEMA than with HEA. Probably the transesterification reaction, transferring the acrylate moiety from the HEA moiety to the end-hydroxyl group of the polymer, Scheme 4 B, competes with the ROP, thus slowing it down. The formation of the HEA/HEMA-initiated polymer, 1, (Scheme 4 A) and the formation of the polymer with acrylated/methacrylated end-hydroxyl groups (3, Scheme 4 B) are shown in Figure 8. In the first hour, the ROP of PDL was the dominating reaction, resulting in HEA- or HEMA-initiated polymers 1 with 50% and 80% of the total amount of HEA and HEMA, respectively. This was followed by a decrease of the HEA (HEMA) end groups in polymer 1 by an acrylate (methacrylate) transfer process to the end-hydroxyl group of polymer 2, generating polymers 3. This suggested that process B in Scheme 4 became dominant, as compared to process A at this point. The drop in the amount of HEA/HEMA end groups roughly coincided with the point of full monomer conversion. The reaction rate of the initiation process and the lactone conversion were found to be very similar for both HEA and HEMA, while the rate of the acrylate transfer reaction was found to be 10-15 times faster, as compared to the rate of the methacrylate transfer reaction. Thus, when using HEMA as the initiator for PDL, the ROP was the major event at low conversions and the methacrylate transfer resulting in polymer 3 was occurring at a moderate rate (5% after 3 h) (Figure 8, Scheme 4 B). On the other hand when using HEA as the initiator, the activity of the acrylate transfer resulted in high levels of polymer 3 even at
an earlier stage of the ROP (50% after 3 h). A similar trend was found when using CL as the monomer, with the difference that the ROP of CL was slower and thus the monomer was available for a longer period of time. Consequently, transacylation reactions with HEA/HEMA also occurred at a lower rate. Nevertheless, the HEA reaction appeared to have reached thermodynamic equilibrium within the reaction time of 70 h, while the slower HEMA transacylation did not (Figure 8 B).

The difference in the acyl-transfer rate between an acrylate and a methacrylate moiety on a polymer is probably due to the combined effects of: a) difference in the monomer-active site complementarity due to the methyl group in the HEMA moiety making it less efficient as an acyl donor; and b) the lower chemical reactivity of the HEMA group in acyl-transfer reactions. It is well known that an α-methyl moiety in acids/esters will lower the chemical reactivity in acyl transfer reactions.64

We used 2-hydroxyethyl (meth)acrylate (HEA and HEMA) as initiators for the ring opening polymerization in order to obtain mono(meth)acrylate functionalized polymers. The ester moieties of the initiators were found to be substrates for CALB. The low selectivity of CALB towards the different acyl donors in the reaction resulted in three processes during the reaction: ROP, (meth)acrylate transfer and polymer chain transfer. As a consequence of these processes a mixture of polymers with different end groups was obtained. The consideration of the enzyme specificity towards all the substrates in the reaction is of importance to design a process avoiding the formation of undesired by-product.
3.3 Substrate selectivity as tool to make polyester (meth)acrylates

In order to overcome the problems of having a mixture of polymers with different end groups when using HEMA as an initiator for ROP and exclusively prepare polymers with two methacrylated ends, we attempted a one-pot procedure with HEMA-initiated ROP reaction ($\alpha$-functionalization) combined with vinyl methacrylate end capping ($\omega$-functionalization) (Scheme 5) (Paper III). Vinyl methacrylate was preferred over other methacrylates since the vinyl group once released will tautomerize to acetaldehyde that evaporates driving the reaction towards the methacrylation. The ROP reaction started by mixing HEMA, the lipase and PDL and it was allowed to run for 24 hours. Then the terminator was added and the reaction was run for further 48 hours. By $^1$H NMR, full conversion of the hydroxyl ends was observed and fully dimethacrylated polymers were obtained.

As presented in the previous section (Paper III and IV) CALB can show simultaneous ROP and transacylation specificities. Based on this fact, we developed a single-step procedure for the synthesis of di(meth)acrylated PPDL (Scheme 6) (Paper VI). The strategy used in this procedure was based on mixing the enzyme with the lactone and a diester carrying two (meth)acrylate groups (ethylene glycol diacrylate (EG diacrylate) or ethylene glycol dimethacrylate (EG dimethacrylate)). In reaction A (Scheme 6), ethylene glycol diacrylate was mixed with PDL and CALB. The reaction was started without any pre-drying as water molecules were needed as nucleophiles (initiators).
The difunctionalization of the produced polyester was mainly due to the transacylation activity of the enzyme. Reduced pressure was applied after 2 hours in order to evaporate water and push the equilibrium towards the difunctionalization. The reaction was allowed to run for 24 hours in total. EG dimethacrylate was used in a similar set up (Reaction B in Scheme 6) and the reaction was allowed to run for 48 hours (since the methacrylate was empirically found to react slower than the acrylate).

According to the $^1$H NMR two types of di(meth)acrylated polymers were detected in reactions A and B; in the first type of polymers, the diol group was located next to the (meth)acrylate group (5, 5') while in the second type the diol group was located within the polyester chain (6, 6'). The presence of the two (meth)acrylate end groups was confirmed and we found by H$^1$ NMR that the fraction of diacrylate ends was 96 % and the degree of dimethacrylation was >95 % (Table 6).

<table>
<thead>
<tr>
<th>D\textsuperscript{a}</th>
<th>Ratio D:M\textsuperscript{b}</th>
<th>Products</th>
<th>Time (h)</th>
<th>Conv. (%)\textsuperscript{c}</th>
<th>(Meth)Acrylate ends (%)</th>
<th>(M_n (\text{Da}))</th>
<th>NMR</th>
<th>SEC</th>
</tr>
</thead>
<tbody>
<tr>
<td>EG diacrylate</td>
<td>1:10 5 and 6</td>
<td></td>
<td>24</td>
<td>&gt;95</td>
<td>96</td>
<td>4000</td>
<td>5800</td>
<td></td>
</tr>
<tr>
<td>EG dimethacrylate</td>
<td>1:10 5' and 6'</td>
<td></td>
<td>48</td>
<td>&gt;95</td>
<td>&gt;95\textsuperscript{c}</td>
<td>3800</td>
<td>6200</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a}D=difunctional diester (ethylene glycol di(meth)acrylate) and M=monomer. The ratio is in mol/mol. \textsuperscript{b}Monomer conversion was determined by NMR. Conversion was estimated using the peak height of the monomer peak at 4.14 ppm. Integration was not possible due to interference with the methylene appearing at 4.15 ppm. \textsuperscript{c}The NMR signal of the methylene group, adjacent to the hydroxyl end of the poly-PDL, was too small to be quantified.

At the beginning of the reaction two acyl donors (lactone and EG di(meth)acrylate) and one acyl acceptor (water) were available in the system. The high selectivity of CALB towards the lactone will drive the ROP towards transacylation of EG di(meth)acrylate. If the enzyme would show low selectivity, (meth)acrylic acid would be produced as a result of hydrolysis of EG di(meth)acrylate and would also lead to a low ratio of functional ends since (meth)acrylic acid is a poor acyl donor.\textsuperscript{75} After ring opening of the lactone, another acyl acceptor will also be available in the system (hydroxyl end of the growing chain) and the (meth)acrylate transfer into the polymer ends will slowly take place. As a result of the (meth)acrylate transfer, two acyl acceptors (2-hydroxyethyl (meth)acrylate and 1,2-ethanediol) will be produced. Reduced pressure was applied in order to have an acyl acceptor exchange and to push the equilibrium towards the formation of (meth)acrylated ends. In other words, when the enzyme is acylated by the free carboxylic end group (formed by the initiation with water), a water molecule will be released and will evaporate by the reduced pressure.
4. Enantioselectivity

Pure enantiomers of chiral compounds have the same physical properties but can cause different responses in biological systems. For example, one enantiomer of ethambutol is used to treat tuberculosis while the other causes blindness. The separation process of the two enantiomers is called resolution. The reason for the enantioselectivity of enzymes is that the enzyme-transition state binding differs between the two enantiomers depending on the complementarity between the active site and the enantiomers. The basis of kinetic resolution is the difference in catalytic efficiency that the enzyme has towards the two enantiomers. This makes enzymes attractive catalysts in the pharmaceutical industry for the resolution of various chiral compounds. In order to increase or invert the enantioselectivity, several enzymes have been subjected to engineering work, both by rational design (Paper V and 76) and by random mutagenesis.77

4.1 Redesigning the enantioselectivity towards secondary alcohols

Like all lipases, CALB shows high enantioselectivity towards the R-enantiomers of secondary alcohols.78 A secondary alcohol has two substituents which are referred to as the medium and large sized substituents. The enantiopreferred configuration fits in the active site by placing the medium sized substituent in a small cavity called the stereospecificity pocket while the large substituent will be directed towards the entrance of the enzyme active site. The other enantiomer will have the opposite orientation of the two substituents where the large one will have to fit into the stereospecificity pocket. The stereospecificity pocket in CALB can accommodate an ethyl group or smaller, which explains the low specificity of the enzyme towards non-preferred enantiomers with a large group longer than an ethyl.79 Engineering of CALB to invert the R-preference into S-preference is highly interesting since the enzymes that have a natural S-preference such as subtilisin have low activity and stability in organic media.80 In Paper V, CALB has been redesigned to catalyze the transacylation reaction of vinyl butanoate with several secondary alcohols. A mutation of one amino acid with a large side chain (Trp104) that blocks the bottom of the stereospecificity pocket into to alanine resulted in inverted enantioselectivity towards some alcohols.
A molecular modeling study was done in order to analyze the conformational space of the mutant enzyme-enantiomer transition state as compared to the wild type. The reaction intermediate between both enantiomers of 1-phenylethanol and butanoyl-CALB was built as tetrahedral intermediates in W104A mutant and WT. The four structures were subjected to molecular dynamics simulations. In the WT enzyme both enantiomers were found to have the same orientation where the medium-sized group (methyl) was pointing into the stereospecificity pocket while the large group (phenyl) was oriented towards the entrance of the active site (Figure 9, left). While the R-enantiomer was found to have all the hydrogen bonds needed for the reaction (Figure 9, top left), the S-enantiomer adopted an unproductive conformation by not forming all the H-bonds needed (Figure 9, bottom left). In the mutant enzyme the R-enantiomer had the same orientation as in the WT (Figure 9, top right). On the other hand the S-enantiomer adopted a different conformation in the mutant as compared to the WT; the large group (phenyl) was pointing towards the new space created in the mutant and the medium group had an orientation towards the entrance. All the hydrogen bonds needed for the catalysis were observed indicating the existence of a productive conformation (Figure 9, bottom right).

The enantioselectivity towards 1-phenylethanol was determined experimentally, and it was changed from a strong R selectivity with wild type CALB to an S selectivity with the W104A mutant, in agreement with the modeling observations. The increased size of the stereospecificity pocket in the mutant facilitated the binding of the S-enantiomer in an orientation conductive to catalysis leading to a higher ratio of productive binding. The apparent kinetic constants ($k_{cat}^{app}$ and $K_M^{app}$), the specificity constant ($k_{cat}/K_M$) and the enantioselectivity ($E$) were calculated for both wild type and W104A mutant for the acylation of (R)- and (S)-1-phenylethanol with vinyl butanoate in cyclohexane at 30 °C (Table 7).
Figure 9. The active site of wild type CALB (left) and Trp104Ala mutant (right) with the butanoate ester of (R)-1-phenylethanol (top) and (S)-1-phenylethanol (bottom) covalently bound to the catalytic serine in the tetrahedral reaction intermediate. The substrate is presented with a stick model and amino acid 104 with a space-filling model in white. The R-enantiomer has a similar configuration in the wild type CALB and Trp104Ala mutant: the large substituent (phenyl) points towards the active-site entrance and the medium-sized substituent (methyl) is positioned in the stereospecificity pocket. In the wild type CALB, the S-enantiomer cannot position its phenyl group in the stereospecificity pocket, and not all the hydrogen bonds required for catalysis can be formed. In the W104A mutant, the phenyl group is comfortably accommodated in the space liberated by the mutation in the stereospecificity pocket (Paper V).
Table 7. Apparent kinetic constants, specificity constants, preferred enantiomer (Pe), and $E$ value of the wild-type CALB and Trp104Ala mutant for the acylation of the pure enantiomers of 1-phenylethanol in cyclohexane at 30 °C with 500 mM vinyl butanoate (Paper V).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate enantiomer</th>
<th>$k_{cat}^{app}$ (s$^{-1}$)</th>
<th>$K_M^{app}$ (mM)</th>
<th>$k_{cat}/K_M$ (s$^{-1}$ M$^{-1}$)$^a$</th>
<th>Pe</th>
<th>$E^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>R</td>
<td>570</td>
<td>0.00053</td>
<td>9300</td>
<td>R</td>
<td>1300000</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>61</td>
<td>71</td>
<td>0.0075</td>
<td></td>
<td></td>
</tr>
<tr>
<td>W104A</td>
<td>R</td>
<td>4.4</td>
<td>29</td>
<td>150</td>
<td>S</td>
<td>6.6</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>34</td>
<td>34</td>
<td>1000</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Calculated from $k_{cat}^{app}$ and $K_M^{app}$.

The enantioselectivity was changed by a factor of 8 300 000, which is higher than any other example found in the literature.

Figure 10. The $S$ selectivity increased with temperature in the resolution of 1-phenylethanol catalyzed by the W104A mutant of CALB in acetonitrile ($\star$), cyclohexane ($\boldsymbol{\star}$), and cis-decalin ($\star$) (Paper V).

The choice of solvent for the reaction can have an effect on the enzymatic enantioselectivity.$^{81-82}$ We performed a study of the solvent influence on the enantioselectivity of the mutant W104A towards 1-phenylethanol at different temperatures. Three different solvents with different molecular sizes were chosen for the study. Acetonitrile, cyclohexane and cis-decalin were used at temperatures between 10 and 70 °C. A trend of increase in enantioselectivity when increasing the solvent molecular size was observed with the highest $E$ value of 44 with $S$ preference at 69 °C (Figure 10). It was suggested by Ottosson et al. that
the number of solvent molecules involved in the solvation of the transition state differs between the two enantiomers since they occupy different spaces in the active site.\textsuperscript{83} Temperature is known to influence the rate of chemical reactions. The effect of temperature on enantioselectivity of enzymatic reactions has been extensively studied. It would be expected that increasing the temperature will lead to a decrease in enantioselectivity.\textsuperscript{84,85} However, an increase in enantioselectivity was observed when increasing the temperature using the W104A mutant. This unusual behaviour has been shown for the acylation of different secondary alcohols: 1-phenylethanol, 1-phenylpropanol and 2-hexanol. Enantioselectivity is caused by a difference in activation energy between the two enantiomers and can be expressed in differential enthalpic ($\Delta_{R,S} \Delta H^\ddagger$) and entropic ($\Delta_{R,S} \Delta S^\ddagger$) terms (Equation 1).

$$\Delta_{R,S} \Delta G^\ddagger = \Delta_{R,S} \Delta H^\ddagger - T \Delta_{R,S} \Delta S^\ddagger = -RT \ln E \quad \text{(Equation 1)}$$

The differential enthalpy and entropy between the enantiomers can be calculated by the linear relation between $\ln E$ and the reciprocal temperature according to Equation 2:

$$\ln E = \frac{\Delta_{R,S} \Delta H^\ddagger}{R} \cdot \frac{1}{T} + \frac{\Delta_{R,S} \Delta S^\ddagger}{R} \quad \text{(Equation 2)}$$

The thermodynamic components were calculated for the W104A mutant with racemic 1-phenylethanol, 1-phenylpropanol, 2-hexanol and 3-methyl-2-butanol (Table 8). For the WT, the thermodynamic components were calculated only for 3-methyl-2-butanol since when using the other alcohols, the $E$ values were too high to be determined accurately. The differential activation enthalpy and entropy values ($\Delta_{S,S} \Delta$) had the same sign for all substrates which indicates that the $S$-enantiomer is favoured by entropy but not by enthalpy. The entropy contribution was larger than the enthalpy which makes the mutant enzyme $S$-selective except for 3-methyl-2-butanol where the enthalpy contribution was larger, leading to $R$-preference. The differential entropy values were similar for all substrates with the mutant but lower in the case of 3-methyl-2-butanol with WT. This suggests that the entropy has accounted for the $S$-preference or low $R$-preference with the mutant. The high entropy effect is caused by the higher degree of freedom gained by the $S$-enantiomer by the additional space created in the mutant. In a new study, it has been shown that the interplay between entropic and enthalpic contributions governs the change in enantioselectivity between the W104A mutant and the WT based on the length of alkyl chains of 1-phenylalkanols.\textsuperscript{86}
Table 8. Thermodynamic components for acyl-transfer reactions from vinyl butanoate to secondary alcohols catalyzed by CALB and the W104A mutant. Differential entropy ($\Delta_{S,R}\Delta S^\ddagger$) and enthalpy ($\Delta_{S,R}\Delta H^\ddagger$) and their standard errors were determined from the linear regression of $R\ln E$ versus $T^{-1}$. The entropic contribution ($T\Delta_S$), Gibbs free energy ($\Delta_{S,R}\Delta G^\ddagger$), and the entioselctivity ($E$) were calculated for 303 K. The preferred enantiomer (Pe) and the racemic temperature ($T_R$) are also presented (Paper V).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Sec. alcohol</th>
<th>Pe</th>
<th>$E^\ddagger$</th>
<th>$\Delta_{S,R}\Delta G^\ddagger$</th>
<th>$T\Delta_{S,R}\Delta S^\ddagger$</th>
<th>$\Delta_{S,R}\Delta H^\ddagger$</th>
<th>$\Delta_{S,R}\Delta S^\ddagger$</th>
<th>$T_R$</th>
</tr>
</thead>
<tbody>
<tr>
<td>W104A</td>
<td>$S$</td>
<td>7.9</td>
<td>-5.2</td>
<td>35</td>
<td>30±2</td>
<td>116±5</td>
<td>260</td>
<td></td>
</tr>
<tr>
<td>W104A</td>
<td>$S$</td>
<td>12</td>
<td>-6.3</td>
<td>26</td>
<td>20±1</td>
<td>87±4</td>
<td>230</td>
<td></td>
</tr>
<tr>
<td>W104A</td>
<td>$S$</td>
<td>2.2</td>
<td>-1.9</td>
<td>23</td>
<td>21±2</td>
<td>77±5</td>
<td>280</td>
<td></td>
</tr>
<tr>
<td>W104A</td>
<td>$R$</td>
<td>2.8</td>
<td>2.6</td>
<td>26</td>
<td>28±2</td>
<td>84±5</td>
<td>330</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>$R$</td>
<td>470</td>
<td>15</td>
<td>7.8</td>
<td>23±2</td>
<td>26±7</td>
<td>900</td>
<td></td>
</tr>
</tbody>
</table>

*Calculated for 303 K from the linear relation of (Equation 2).

In conclusion, we showed that by a single amino acid mutation the enantioselectivity was changed by 8'300'000 times as compared to the WT. This indicates how sensitive the selectivity can be to small changes in the enzyme structure. Furthermore, the reaction rate for the S-enantiomer increased 64'000 times in the mutant as related to the WT. The data presented shows clearly how the entropy could be of importance for changing the specificity towards an enantiomer. The entropy caused the increase in $E$ with higher temperature. In addition, solvent had a large influence on the selectivity.
5. Chemoselectivity

Enzymatic chemoselectivity takes place when an enzyme shows a different specificity towards one of several functional groups presented in the same reaction. In Paper VI and VII we took advantage of the chemoselectivity of CALB for the synthesis of end functionalized polyesters.

5.1 Enzymatic synthesis of end-functionalized polyesters

Polyesters are a type of degradable polymers relevant in the field of biomaterials. The combination of biocompatibility and biodegradability with the physical strength provided by the polymer is critical for such applications. However, the preparation of end-functionalized polyesters will allow making further chemical modification in order to obtain more complex polymer structures and architectures. Enzyme catalysis offers novel methodology for single-step synthesis of end-functionalized polyesters. Functional groups can be introduced enzymatically to a polyester chain either by using an initiator that carries a functional group (α-functionalization) or by using a terminator (end capper) (ω-functionalization). Both the initiator and terminator could be combined together to give difunctionalized chain (α,ω-difunctionalization) (Paper VI and VII).

α-Functionalization (by initiation method)

In the α-functionalization approach the functional end is introduced by using a functional initiator (nucleophile that carries an additional functional group) which will start the ROP of the lactone. Many functional end groups have been introduced by α-functionalization method as showed by Cordova et. al. and in (Paper VI and VII). However, there are some limitations in using the enzymatic α-functionalization method of polyesters, for example using functional initiators with cleavable ester bond such as 2-hydroxyethyl (meth)acrylate (HEA and HEMA) (Paper III and IV).

ω-Functionalization (by termination method)

By ω-functionalization, a functional end group is introduced using an acyl donor (end capper) carrying a functional group. The end capper can be a carboxylic acid, an ester or a lactone (Paper VI and VII).
Enzymatic synthesis of polymers with two thiol groups

Polymers thiol-capped at one or both ends are of interest and have been used in biochemistry, materials science,\textsuperscript{90} and in the addition of species to gold.\textsuperscript{91} Hedfors et al. used thiol functional initiators or terminators for $\alpha$ or $\omega$-end functionalization of $\varepsilon$-caprolactone.\textsuperscript{54} In Paper VI and VII, we introduced $\alpha$- and $\omega$-end functionalities simultaneously to one polymer molecule using CALB. The high chemoselectivity of CALB towards alcohols over thiols as nucleophiles allowed the use of mercaptoalcohols as a source of thiol functionality on the polymers. When using a mercaptoalcohol as an initiator for ROP ($\alpha$-functionalization), mainly the alcohol group acted as a nucleophile and the thiol end remained intact. To introduce a thiol end by $\omega$-functionalization a mercaptoacid or thiolactone can be used. In Paper VII, 6-mercapto-1-hexanol (Scheme 7) was used as an initiator for the ring opening polymerization of $\omega$-pentadecalactone (Route A in Scheme 7).

The reaction was allowed to run for 20 hours before $\gamma$-thiobutyrolactone was added as a terminator. In order to speed up the reaction we used an excess of the thiolactone, since $\gamma$-thiobutyrolactone contains a thioester group, which has been shown to be slower in a transesterification reaction than an oxy ester.\textsuperscript{92} The advantage of using $\gamma$-thiobutyrolactone is that no coproduct could be formed. About 97% of the polymers had thiol group in the $\alpha$-end and 92% had thiol group in $\omega$-end (product 7, Table 9). This high percentage is of great importance since the functional ends must be as quantitative as possible in order to have good control when using the polymers for making further architectures.

Since CALB has the ability of catalyzing ROP and transacylation reactions simultaneously as shown in Paper III and IV, we developed a single-step route for the synthesis of $\alpha,\omega$-thiol functionalized PPDL polymers (Route B, Scheme 7). The initiator 6-mercapto-1-hexanol and the terminator 11-mercapto-1-undecanoic acid were mixed (without any pre-drying) with $\omega$-pentadecalactone and the lipase (Route B in Scheme 7).
Scheme 7. Difunctionalization of poly-PDL with two thiol ends. Route A: Polymerization of PDL initiated by 6-mercapto-1-hexanol for 20 h and terminated by γ-thiobutyrolactone for 24 h resulting in product (7) with m=2. Route B: Single step synthesis of poly-PDL with two thiol end groups (7) with m=9, by mixing 6-mercapto-1-hexanol and 11-mercapto-1-undecanoic acid with PDL (Based on results presented in Paper VI and VII).

No excess of the terminator was added and the ratio initiator/terminator was 1:1. The reaction was run for 24 hours under reduced pressure to evaporate the produced water. We found by H\textsuperscript{1} NMR analysis of product 7 that both the initiation, with 6-mercapto-1-hexanol, and termination, with 11-mercapto-1-undecanoic acid, were performed with an efficiency of 95 % (Table 9).

The reaction starts with one acyl acceptor (6-mercapto-1-hexanol) and two acyl donors (lactone and 11-mercapto-1-undecanoic acid). Simultaneous ROP and transacylation combined with high chemoselectivity led to the synthesis of difunctionalized polymers. A balanced selectivity of CALB towards both acyl donors is important for the functionalization since if the enzyme has a too high selectivity for the lactone no acylation will occur with the terminator molecules. On the other hand, if the enzyme has a too high selectivity towards the terminator, no ring opening will take place. Reduced pressure was applied in order to push the equilibrium towards the ω-functionalization by evaporating the water molecules when released as a leaving group of the acylation of the enzyme with the terminator.
The high yield of thiol ends gives products suitable for manufacturing of new materials. In fact, the produced polymers (PPDL macromonomers with two thiol ends) were used together with norbornene functional ene-monomers to make semi-crystalline polymer networks using thiol-ene based chemistry.\textsuperscript{93} A simple single-step and solvent free process is of a great interest for industrial applications since a pure product without using an excess of terminator compared to initiator can be afforded.

**Enzymatic synthesis of polymers with thiol and acrylate end groups**

Thiol-ene photopolymerization has several unique properties in being relatively uninhibited by oxygen,\textsuperscript{94} affording rapid solvent free polymerization, enabling radical polymerization of a wide range of thiol and vinyl functional groups and yielding optically clear products.\textsuperscript{95} In addition, thiol-ene chemistry has found applications in areas such as dentistry, surface grafting, coating, adhesives and optical lenses as reviewed by Hoyle et al.\textsuperscript{96} Thiol-ene polymerization is based on the radical-catalyzed addition of a thiol to a vinyl functional group. Thiol-acrylate polymerization is a type of thiol-ene photopolymerization. Polymers with both thiol and acrylate functional groups have great potential in the synthesis of polymer networks.

The high reaction specificity of CALB towards transacylation over Michael-type addition between a thiol and a acrylate allowed the combination of thiol and acrylate groups on the same polymer chain using enzymatic $\alpha,\omega$-functionalization of PDL (Paper VII) (Route A in Scheme 8). Such a reaction has not been reported using a chemical catalyst due to the inherent reactivity of the groups. $\omega$-Pentadecalactone was initiated with 6-mercapto-1-hexanol and the reaction was allowed to run for 20 hours. In a second step, vinyl acrylate as a terminator

\begin{table}[h]
\centering
\caption{Synthesis of di-functionalized poly-PDL with two thiol ends catalyzed by \textit{Candida antarctica} lipase B.}
\begin{tabular}{cccccc}
\hline
$T$ & Product Ratio & Reaction time (h) & Fraction of ends (%) & $M_n$ (Da) \\
\hline
A & 7 (m=2) & 1:5:25 & 20 & 97 & 1500 \\
B & 7 (m=9) & 1:5:1 & 24 (one step) & 95 & 2400 \\
\hline
\end{tabular}
\begin{tabnote}
a I= initiator, T= terminator, M= monomer. The ratio is in mol/mol. b A= $\gamma$-thiobutyrolactone, B= 11-mercapto-1-undecanoic acid. c Values obtained from size exclusion chromatography.
\end{tabnote}
\end{table}
was added in fifteen times excess to initiator. The presence of one thiol and one acrylate end in product 8 (Scheme 8) was confirmed by $^1$H NMR spectroscopy and 85% of the polymers contained a free thiol and over 95% were acrylate terminated (Table 10).

Michael-type addition between some of the thiol ends and the acrylic group of the vinyl acrylate was detected by $^1$H NMR. A study done by Carlqvist et al. has shown that CALB can catalyze the Michael-type addition between a thiol and an acrylic group.97 The Michael-type addition could explain the drop of the thiol end groups in polymer 8 (Scheme 8) compared to polymer 7 (Scheme 7).

A single-step route for the synthesis of $\alpha,\omega$-functionalized PPDL with thiol and acrylate was developed (Route B, Scheme 8). The initiator 6-mercapto-1-hexanol and the terminator vinyl acrylate were mixed with PDL and CALB. The reaction was run for 6.5 hours and no excess of the terminator was used. To prevent any loss of vinyl acrylate, no reduced pressure was applied. The enzyme, initiator and lactone were dried prior to start to avoid any nucleophilic competition with water.
The presence of thiol and acrylate functional ends in product 8 was confirmed by $^1$H NMR and 86 % of the polymers were thiol initiated and 96 % were acrylate terminated (Table 8). As already discussed in the last paragraph, the simultaneous ROP and transacylation combined with high chemoselectivity led to the synthesis of difunctionalized polymers. No reduced pressure was applied in this reaction since the vinyl acrylate has a low boiling point and might be evaporated. The leaving group, vinyl alcohol, will tautomerize and leave the reaction as described in section 3.3. The single-step route for synthesis of difunctional PPDL (Paper VI) showed an advantage over the two-step procedure (Paper VII) as the single-step route used equimolar ratio of initiator and terminator and afforded a cleaner product.

**Table 10.** Synthesis of difunctionalized poly-PDL with one thiol and one acrylate end catalyzed by *Candida antarctica* lipase B.

<table>
<thead>
<tr>
<th>Product</th>
<th>Ratio I:M:T</th>
<th>Reaction time (h)</th>
<th>Fraction of ends (%)</th>
<th>$M_n$ (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>1:5:15</td>
<td>20</td>
<td>85</td>
<td>&gt;95</td>
</tr>
<tr>
<td>8</td>
<td>1:5:1</td>
<td>6 (one step)</td>
<td>86</td>
<td>96</td>
</tr>
</tbody>
</table>

* $I= $ initiator, $M= $ monomer and $T= $ terminator. The ratio is in mol/mol. Values obtained from size exclusion chromatography.
6. Conclusions

Rational redesign of *Candida antarctica* lipase B for two different applications was successfully conducted. A catalyst with an improved activity towards the polymerization of $D,D$-lactide was obtained by a single mutation. This enzyme variant could be the first step towards the design of a catalyst for industrial use for polymerization of lactides. A second catalyst with inverted enantioselectivity towards a few secondary alcohols was successfully achieved by a single mutation. Creating an $S$-selective lipase is of a good potential for pharmaceutical applications. Yet, the stability and activity of this mutant is to be optimized for industrial scale application.

The importance of the consideration of substrate selectivity when designing an enzymatic reaction was shown in this thesis. When a molecule carries two reactive sites for the enzyme, a mixture of products can be obtained depending on the specificity of the enzyme towards each of these sites. The use of $\gamma$-acyloxy-$\varepsilon$-caprolactone monomers to obtain pendant functional polymers was possible only when the enzyme showed a high selectivity towards the lactone ester over the $\gamma$-ester. The size of the $\gamma$-ester group determined the selectivity; with a large $\gamma$-ester group only polymerization could occur while with a small one the substrate rearrange to $\gamma$-acetyloxyethyl-$\gamma$-butyrolactone. The use of a functional initiator molecule carrying an ester moiety, such as HEA and HEMA, for ROP showed to be of a limited applicability since the ester moiety was a substrate for the enzyme. The enzyme simultaneously catalyzed the ROP and the transacylation of the ester moiety resulting in a mixture of polymers with different end groups. It was not possible to obtain polymers with mono(meth)acrylated ends using HEA or HEMA. However, combining those initiators with a proper end capper ester made it possible to obtain difunctionalized polymers.

High chemoselectivity showed by CALB towards alcohols over thiols as nucleophiles made it possible to synthesize mono- or di-thiol end functionalized polyesters. The high reaction selectivity displayed by CALB towards transacylation as compared to Michael-type addition helped in combining thiols and acrylates on the same polymer chain. Taking advantage of the ability of CALB to catalyze simultaneous ROP and transacylation, we developed a simple single-step route for the synthesis of difunctionalized polyesters. Such a route could be of great industrial interest.
7. Acknowledgments

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Neil Simpson thanks for being a good friend inside and outside the lab. For all the good fun at the meeting in Paris and especially the excellent drawings during the conference, they made some lectures less boring and as I have promised you, I will have one in my thesis so see Figure XXX.

Figure XXX: From the right: Neil Simpson, Magnus Ericsson and Mohamad Takwa.
Magnus Ericsson for teaching me to have two lunches per day! Thanks for making lunch time at the lab more human and for all the fun in the lab and for being a good friend.

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أخيرا أتوجه بالشكر إلى جميع الأهل والأصدقاء والأخص: الوالد الغالي والوالدة الحبيبة، جزاك الله خير على تعبكم معي ودعمكم إلي، الله يبارك بأعمري ويخليننا إياكم. الزوجة الغالية دانة وابني الحبيب أبو حفص أخوتى: عبودة، أحمد، مروة و أبو فارس أبو حميد رواحنة الحبيب الاستاذ ياسر علي ديب الغالي أبو الفوز الورد والأخت زينة الأخ و الجار العزيز حسين الأخ كميل الغالي


8. References


