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Template-assisted enzymatic synthesis of oligopeptides from a polylactide chain

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ABSTRACT

Peptides are often attached to polymer materials, as bioactive components, for the control of interactions between the material and its surrounding proteins and cells. However, synthesizing peptides and attaching them to polymers can be challenging and laborious. Herein, we describe the grafting of oligopeptides to an aliphatic polyester, using a one-step chemo-enzymatic

synthesis with papain as the biocatalyst. To enable enzyme-mediated functionalization of the polyester, ethyl hept-6-enoylalaninate (grafter) was synthesized and attached to polylactide chains using thiol-ene click reactions. The oligopeptides were grafted onto the polylactide chains using two different synthetic routes: the grafting from strategy, in which the grafter was attached to the polyester prior to oligopeptide synthesis, or the grafting to strategy, in which oligopeptides were synthesized on the grafter first, then attached to the polymer chain. The final products were analyzed and their structures were confirmed using nuclear magnetic resonance (NMR). The peptide attachment was evaluated using size exclusion chromatography (SEC), contact angle measurement and energy-dispersive X-ray spectroscopy-scanning electron microscopy (EDS-SEM). Furthermore, the mechanistic aspects of the synthesis of the oligopeptides on the grafter were studied using molecular dynamics (MD) simulations. The simulation revealed that hydrogen bonding (between the P1 amide nitrogen of the grafter backbone and the carbonyl oxygen of D158 in the papain) maintain the grafter in a productive conformation to stabilize the transition state of nitrogen inversion, a key step of the biocatalytic mechanism. Apart from being biologically relevant, both experimental and computational results suggest that the designed grafter is a good template for initiating chemo-enzymatic synthesis. The results also showed that the grafting to strategy was more successful compared to the grafting from strategy. Overall, a successful synthesis of pre-defined peptide functionalized polylactide was prepared, where the oligopeptides were grafted in an easy, time efficient and environmentally friendly way.

Introduction

Over the last few decades, aliphatic polyesters have been used as scaffold materials for tissue engineering.[1–4] Lately, peptide functionalization of aliphatic polyesters has been of great interest because of the desire to control the interactions between materials and their surrounding tissue

cells (e.g., to reduce inflammatory responses and to stimulate cell differentiation). Many different types of copolymers have been designed.[5–7] A promising way to vary the properties of these copolymers is to introduce functional groups onto the main chain (e.g., epoxy, vinyl and thiol groups) and, in turn, modulate the surface properties through protein attachment. For example, J. Undin *et al*. functionalized a polyester-based copolymer with epoxy groups that were then used to attach heparin.[8] In a study conducted by S. Suliman, scaffolds of poly(L-lactide-co-ε-caprolactone) were immobilized by a low dose of bone morphogenic protein-2 by four different functionalizing techniques.[9] Using thiol groups and thiol-ene click chemistry is another versatile method for the functionalization of polymers. In recent study conducted by our group, T. Fuoco *et al*. synthesized poly(L-lactide) with pendant thiols, which were then covalently linked through a disulfide exchange reaction with arginine-glycine-aspartic-cysteine acid peptide sequences.[10]

Proteins are usually prepared from solid-phase peptide synthesis (SPPS) or recombinant DNA techniques. On one hand, SPPS constitutes an excellent technique for designing specific proteins, but on the other hand it is expensive, requires toxic chemicals and is time consuming.[11,12] Furthermore, synthesizing shorter peptides using established biotechnological methods remains a challenge.[13] Chemo-enzymatic synthesis offers a nontoxic and simple method for generating peptides.[14–18] The technique can either be thermodynamically or kinetically controlled; the latter method is more commonly used since it is selective and generally gives higher yields.[19] Many different enzymes can serve as biocatalysts for peptide synthesis. The most studied are proteases (which naturally hydrolyze peptide bonds) but under the right conditions (e.g. pH, temperature and substrate concentration) can also catalyze peptide bond formation to produce

polypeptides.[20–22] Examples of common proteases used in chemo-enzymatic syntheses are α-chymotrypsin, bromelain and papain.[15,20,23]

The advantages of chemo-enzymatic synthesis (being a simple synthesis method, performed in mild reaction conditions, i.e. natural pH and low pressure and temperature) make it an interesting technique to graft peptides to a polyester chain. Studies using papain to functionalize polymers has been reported earlier, where co-oligomers of leucine and nylon esters were synthesized to obtain thermal processable peptides.[24]

In order to grow peptides from a synthetic molecule it is essential to understand the catalytic mechanism of the enzyme. This can be done by molecular dynamics (MD) simulations, where the tetrahedral intermediates (representing the rate-limiting transition state (TS) for amide bond formation) are analyzed. Amino acids of interest for cell stimulation include for example; L-lysine [K], L-serine [S] and L-arginine [R]). In particular they have different properties in terms of their polarity, charge and ability to promote different types of cell interactions [25–27].

With this project we sought to functionalize an aliphatic polyester with oligopeptides using enzymatic synthesis and papain as biocatalyst. Papain was chosen for its broad substrate specificity and for its ability to operate in a broad pH range (4-8).[28,29] However, it should be noted the substrate acceptance and esterase activity of papain might hydrolyze not only peptides but also polyester bonds. The oligopeptides were synthesized and grafted onto an aliphatic polylactide main chain by two different grafting strategies; the grafting from strategy and the grafting to strategy (Scheme 2). To carry out the oligopeptide functionalization of the polyester, a grafter (ethyl hept-6-enoylalaninate) was prepared. The design of the grafter was made by

taking in to account the mechanism for enzyme-catalyzed peptide synthesis provided by MD simulations.

Experimental

Materials

All chemicals were used as received, unless otherwise stated. L-lactide (LLA) was purchased from Boehringer Ingelheim (Germany), purified by recrystallization in dry toluene and then dried *in vacu*o. 3-Methyl-6-(tritylthiomethyl)-1,4-dioxane-2,5-dione (TrtS-LA) was synthesized according to the procedure reported by T. Fuoco *et al*.[10] Dry tetrahydrofuran (THF) and dry dichloromethane (CH_2Cl_2) were purchased from Sigma-Aldrich; all other solvents were purchased from Fisher Scientific (USA). Triethylamine (NEt_3), 6-heptenoic acid, thionyl chloride ($SOCl_2$), trifluoroacetic acid (TFA), triethylsilane ($SiHEt_3$), stannous octoate ($Sn(Oct)_2$), 2,2′-azobis(2-methylpropionitrile) (AIBN), ethyl 6-heptenoate, L-alanine ethyl ester (A-Et), L-lysine ethyl ester (L-Et), L-serine ethyl ester (S-Et), L-arginine ethyl ester (R-Et) and papain (Latex papaya,10 units/mg protein) were purchased from Sigma-Aldrich (Germany).

Methods

Nuclear magnetic resonance (NMR)

A Bruker Advance DPX-400 NMR operating at 400 MHz with an autosampler was used to elucidate the molecular structure of the samples. The analyses were performed in either deuterated chloroform or deuterium oxide using tetramethylsilane (TMS) as an internal standard.

Size exclusion chromatography (SEC)

The number average molecular weight (Mn) and dispersity (Đ) of the polymeric films grafted with oligopeptides were analyzed using size exclusion chromatography (SEC) in dimethylformamide (DMF) with 0.01 M LiBr as the mobile phase with a flow rate of 0.2 ml/min at 35°C. A TOSOH EcoSEC HLC-8320 GPC system from PSS GmbH with three columns (PSS PFG 5 mm; Microguard, 100 Å and 300 Å) (MW resolving range: 300-300 000 Da) was used, and the detector was an EcoSEC RI. Narrow linear polyethylene glycol (PEG) standards purchased from PSS with a range of 106-44000 Da were used for calibration. Corrections of the flow rate fluctuations were carried out with toluene as the internal standard. PSS WinGPC Unity software version 7.2 was used to analyze the data. Samples with an approximate concentration of 2.5 mg/ml in DMF were prepared and filtered before the analysis.

Contact angle measurement

The surface properties of poly(L-lactide-*co*-thiol-lactide) grafted with oligoalanine (grafter-oligoA), oligolysine (grafter-oligoK), oligoserine (grafter-oligoS) and oligoarginine (grafter-oligoR) were analyzed using a CAM 200 contact angle system. Films of the samples were prepared from 10 mg/ml solutions of the sample in chloroform, which were cast on optical glass slides (Ø: 13 mm) and positioned in front of an optical camera. A droplet of MilliQ water was placed on the sample films, and the contact angles were measured. The recorded frames were evaluated using CAM 2008 software to calculate the contact angles. The average values were based on three independent analyses for each sample.

Energy-dispersive X-ray spectroscopy-scanning electron microscopy (EDS-SEM)

Films of polylactide-*graft*-oligolysine and polylactide-*graft*-oligoarginine were analyzed using EDS-SEM to study the nitrogen distribution on the surface. The films where coated with 4 nm of platinum using an automatic sputter coater (Agar Scientific, Stansted, UK). An ultra-high-resolution SEM (Hitachi S-4300) equipped with an X-MaxN 80 Silicon Drift Detector (SDD) (Oxford Instruments, USA) was used to obtain the images and to analyze the elements at the surface. An acceleration voltage of 10 kV was used in the analysis, and images were taken at 500 times magnification.

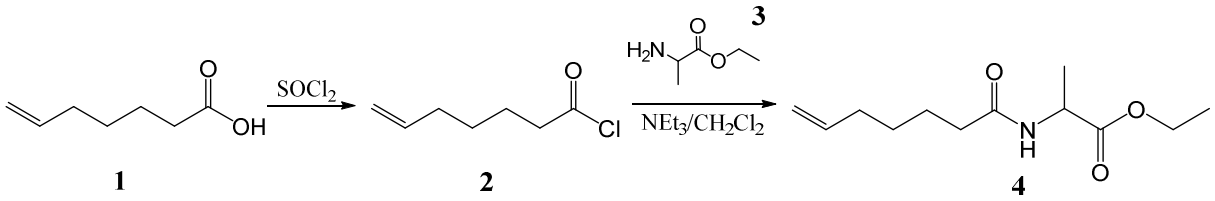
Synthesis of ethyl hept-6-enoylalaninate (grafter)

6-Heptenoic acid (1) was dissolved in $SOCl_2$ (1: 7.5, 6-heptenoic acid: $SOCl_2$) and stirred. The reaction was heated to 80 °C under reflux conditions for 90 min. Unreacted $SOCl_2$, HCl and SO_2 were evaporated using a double trap, and 6-heptenoyl chloride (2) was obtained as a brown oil. In the second step, 3.2 g of 6-heptenoyl chloride (22 mmol) was added dropwise to 3.4 g (22 mmol) of A-Et (3), which was dissolved in dry CH_2Cl_2 (under nitrogen), and the mixture was cooled to 0 °C. As the reaction was stirring, NEt_3 (14 ml, 101 mmol) was slowly added, and the reaction was maintained at room temperature for 24 h. The grafter (4) was separated from NEt_3, HCl and unreacted A-Et using liquid-liquid extraction, where $NaHCO_3$, HCl (1 M), brine and deionized water were sequentially used as extraction solvents. The organic phase (CH_2Cl_2) was dried with anhydrous magnesium sulfate, which was removed by filtration. Organic solvents were removed by rotary evaporation to afford the desired product. The reaction scheme is presented in scheme 1 below.

To confirm a successful synthesis, the grafter was analyzed using ^{1}H-NMR, ^{13}C-NMR, COSY- and HSQC-NMR. ^{1}H NMR (400 MHz, Chloroform-*d*): δ 5.88 – 5.74 (m, 1H), 5.07 – 4.94 (m,

2H), 4.60 (p, *J* = 7.2 Hz, 1H), 4.22 (q, *J* = 7.1 Hz, 2H), 2.24 (t, 2H), 2.09 (q, *J* = 7.1 Hz, 2H), 1.68 (p, *J* = 7.2 Hz, 2H) 1.46 (p, *J* = 7.6 Hz, 2H), 1.42 (d, *J* = 7.1 Hz, 3H), and 1.30 (t, *J* = 7.1 Hz, 3H).

^{13}C NMR (101 MHz, Chloroform-*d*): δ 173.27 (-C(O)OCH$_2$CH$_3$), 172.45 (-C(O)NH-), 138.44 (-CHCH$_2$-), 114.69 (CH=CH-), 61.50 (-CH$_2$CH$_3$), 47.98 (-CHCH$_3$-), 36.38 (-CH$_2$C(O)-), 33.43 (-CH$_2$CH$_2$CH$_2$CH$_2$-), 28.42(-CH$_2$CH$_2$CH$_2$-), 25.02 (-CH$_2$CH$_2$C(O)-, 18.66 (-CH$_3$CH-), and 14.13 (-CH$_3$).



Scheme 1. Synthesis of ethyl hept-6-enoylalaninate (grafter).

Synthesis of poly(TrtS-LA-*co*-LA) and deprotection of the thiol groups

Poly(L-lactide-*co*-3-methyl-6-(tritylthiomethyl)-1,4-dioxane-2,5-dione), [poly(LLA-*co*-LA-TrtS)] was synthesized according to the procedure described by T. Fuoco *et al*.[10] In a glovebox, a round-bottomed flask was charged with LLA, TrtS-LA (85:15), Sn(Oct)$_2$ (24 mg, 59 μmol) and ethylene glycol (4 mg, 60 μmol). The flask was sealed with a rubber septum and heated under stirring at 110 °C for 40 h. The polymer was purified by precipitation in a solution of methanol and heptane (1:9).

The poly(LLA-*co*-LA-TrtS) was dissolved in dry CH_2Cl_2 in a 100 ml Schlenk flask. SiHEt$_3$ (0,815 mmol) was added to the solution under a nitrogen flow, then TFA was added dropwise (1.9 mmol). The solution was kept at room temperature under stirring for 20 h. The solvents were then evaporated using a double trap, and the resulting poly(L-lactide-*co*-thiol-lactide) was washed twice with heptane and stored under nitrogen.

Synthesis of the peptides grafted onto poly(L-lactide-*co*-thiol-lactide)

The grafting from strategy

Attachment of the grafter to poly(L-lactide-*co*-thiol-lactide). Poly(L-lactide-*co*-thiol-lactide) was dissolved in dry THF, and the grafter was added (1:1.6 poly(L-lactide-*co*-thiol-lactide): grafter) under a nitrogen atmosphere. The reaction was initiated using 5 mol% of AIBN, which was added under a flow of nitrogen. The reaction was heated to 80 °C and stirred for 24 h. The product (poly(L-lactide-*co*-lactide-grafter)) was washed with cold heptane and isolated by filtration.

Chemo-enzymatic synthesis of the poly(L-lactide-*co*-lactide-grafter) films. Films of poly(L-lactide-*co*-lactide-grafter) were prepared in 3 ml glass vials. Poly(L-lactide-*co*-lactide-grafter) (20 mg) was dissolved in 1 ml of chloroform. The solution was added to a vial, and the solvent was allowed to evaporate, forming a film at the bottom of the vial. Solutions (0.5 M) of the amino acid ethyl ester of either alanine, lysine, serine or arginine were dissolved in 2 ml of a phosphate buffer solution (PBS, 1 M, pH 7.6). The solutions were added to the poly(L-lactide-*co*-lactide-grafter) films and heated to 40 °C. Papain (21 mg) was dissolved in 1 ml of PBS, then added to the reaction mixture. The solution was kept at 40 °C and allowed to react for 18 h. The films with the attached peptides were then washed once with 0.5 ml of PBS and then twice with 1 ml of deionized water. The films were then left to dry in a fume hood. The films were characterized using ^{1}H-NMR, COSY-NMR and DMF-SEC.

The grafting to strategy

Chemo-enzymatic synthesis on the grafter

The chemo-enzymatic synthesis on the grafter was carried out in a similar way to the previously described enzymatic synthesis on the films. The grafter (0.5 mmol), 1 mmol of amino acid ethyl ester (1:2 grafter: amino acid ethyl ester) and 2 ml of 1 M PBS (pH 7.6) was added to a 3 ml glass vial and preheated under stirring to 40 °C. Papain (21 mg, 7 mg/ml) was then dissolved in PBS and added to the reaction mixture. The temperature was kept at 40 °C, and the reaction was allowed to proceed for 18 h. The reaction was then stopped by the addition of 1 ml of 2 M HCl, which lowered the pH of the solution and deactivated the enzyme. The product (grafter-oligopeptide) was purified from the unreacted grafter by centrifugation (3000*g for 20 min). The supernatant was then removed, frozen to -80 °C and lyophilized. The enzyme and homo-oligomers that remained were removed later in the reaction when the grafter-oligopeptide was attached to the copolymer. The product was analyzed by ^{1}H-NMR.

Attachment of the grafter-oligopeptide to poly(L-lactide-*co*-thiol-lactide)

The oligopeptides synthesized on the grafter by chemo-enzymatic synthesis were attached with thiol-ene click chemistry using AIBN as the radical initiator. The grafter-oligopeptides and poly(L-lactide-*co*-thiol-lactide) were dissolved in dry THF (1:1.6) under a flow of nitrogen. The reaction was initiated by the addition of 5 mol% of AIBN and was heated to 80 °C while stirring. The solution was allowed to react for 24 h. The product was analyzed using ^{1}H-, COSY-NMR, contact angle analysis and EDS-SEM.

Chemo-enzymatic synthesis from ethyl 6-heptenoate

The enzymatic synthesis from ethyl 6-heptenoate was carried out in a similar way as the grafter. A-Et and ethyl 6-heptenoate were dissolved in 2 ml of PBS (1 M, pH 7.6) and preheated to 40 °C while being stirred. Papain was then dissolved in 1 ml of PBS and added to the solution. The

reaction was stirred and heated for 18 h. The reaction was stopped after adding 1 ml of 2 M HCl, and the unreacted ethyl 6-heptenoate and oligoalanine were removed after centrifugation (3000*g for 20 min). The supernatant was then removed from the precipitate, frozen, lyophilized and analyzed using ^{1}H-NMR.

Molecular dynamics (MD) simulations

MD simulations on papain (papaya proteinase I from *Carica papaya*) were based on the X-ray crystal structure complexed with leupeptin (PDB ID code 1POP)[30] using YASARA[31] (Yet Another Scientific Artificial Reality Application) version 16.2.23. All missing hydrogens were added to the starting enzyme structure, and the hydrogen network was optimized using the Amber14 force field. Models of the tetrahedral intermediates were constructed using two different grafters (ethyl hept-6-enoylalaninate and ethyl 6-heptenoate (figure 1)) in the P1 position according to the Schechter and Berger nomenclature[32]and the four respective amino acid monomers (A-Et, S-Et, K-Et and R-Et) at the P1' position. Here, the co-crystallized ligand was used as a template to construct the respective substrates. For the model with ethyl 6-heptenoate, only A-Et was built into the P1' position. Crystallographic water molecules were kept in the simulation. The structure was energy minimized by steepest descent and simulated annealing, initially on all the hydrogens with remaining atoms fixed, and finally on all atoms. The resulting structure was subjected to a short (10 ps) molecular dynamics, followed by an additional round of minimizations. Force field parameterization for the substrates was conducted using the AUTOSMILES[33] methodology. All simulations were performed in a water box that contained approximately 5000 explicit water molecules. The pH was set to 7.6, and the adequate protonation states of the enzyme side chains were predicted using the empirical method built in to YASARA.[34] The simulation cell was neutralized through the addition of 0.9 % NaCl. The MD

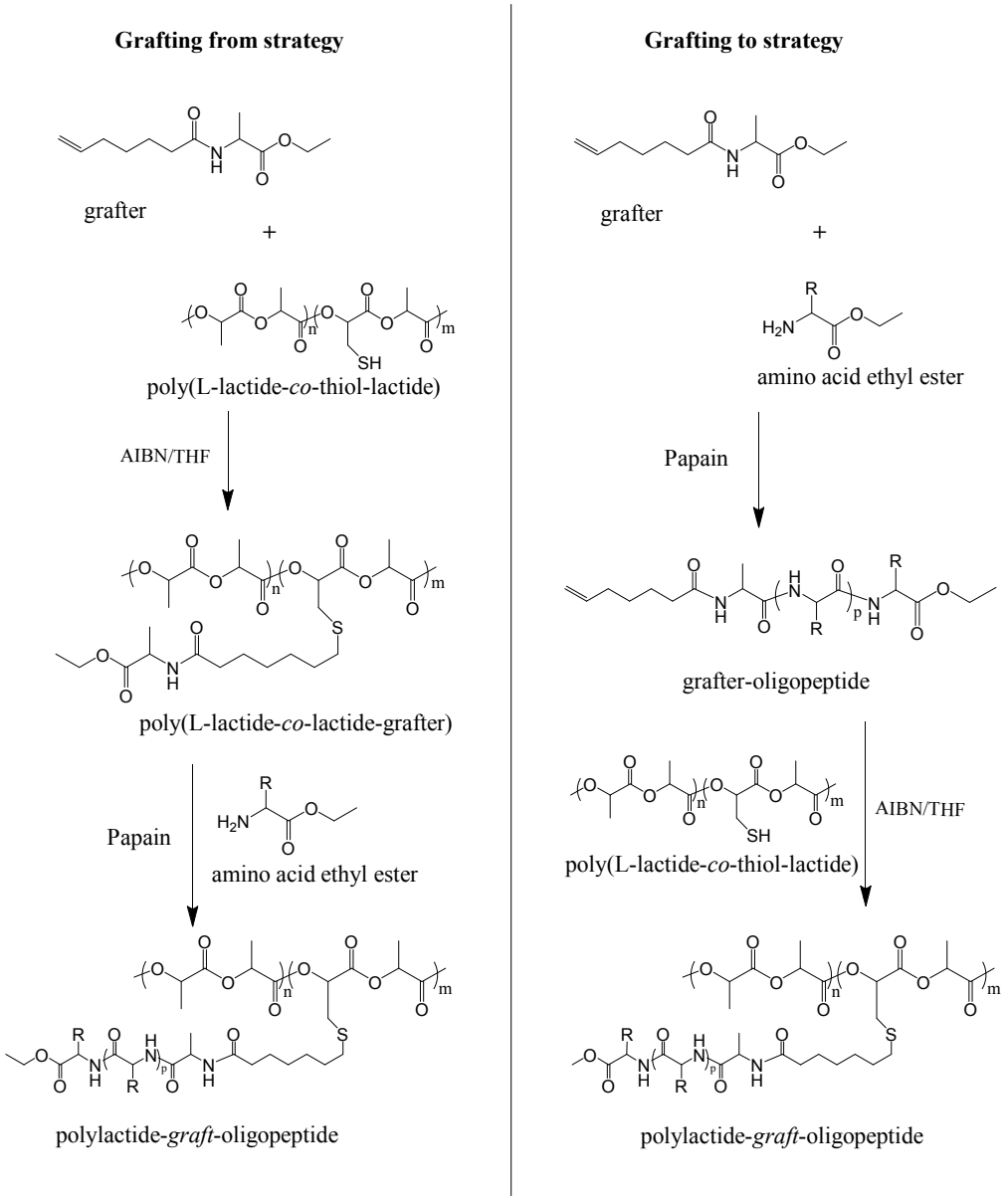
simulations were performed using the canonical ensemble at 313 K with a Berendsen thermostat (and the Amber14 force field). The Particle mesh Ewald (PME) method accounted for long-range electrostatics[35] during the MD simulations, and the cut-off for the van der Waals interactions was set to 8 Å. MD simulations were run for 180 ns with time steps set to 1 fs (the molecular forces were calculated every second substep), saving snapshots every 2.5 ps.

Results and Discussion

Synthetic strategies for polylactide-*graft*-oligopeptide

To graft oligopeptides onto poly(L-lactide-*co*-thiol-lactide), an alanine ethyl ester with a hydrophobic tail (ethyl hept-6-enoylalaninate, grafter) was designed and synthesized. The molecular structure of the grafter is essential in order for the enzyme to recognize it and accommodate it in a productive conformation, to enable peptide synthesis by reaction with the catalytic dyad. It is well known that papain prefers hydrophobic groups in the P2 position and therefore an alanine ethyl ester was chosen as the end group of the grafter.[30]

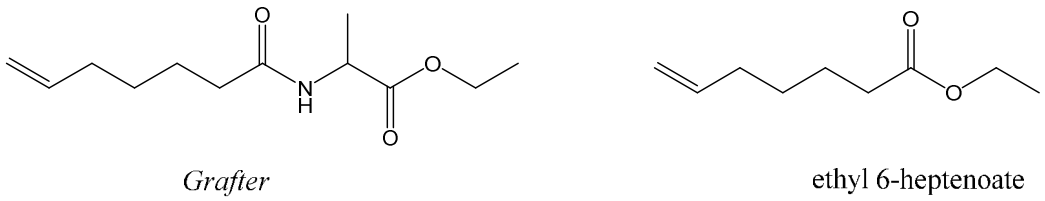
The grafter was then used in two different synthetic routes to obtain peptide-functionalized polylactide (Scheme 2). The first synthetic route is an example of the *"grafting from strategy"*, in which the grafter was attached to poly(L-lactide-*co*-thiol-lactide) through thiol-ene click chemistry (using AIBN as the radical initiator). In the next step, oligopeptides were synthesized from films of poly(L-lactide-*co*-lactide-grafter) using chemo-enzymatic synthesis, producing the desired polylactide grafted with oligopeptides (polylactide-*graft*-oligopeptide). In the second route, a *"grafting to strategy"* was applied, in which the amino acid ethyl esters were added to the grafter by chemo-enzymatic synthesis. After, grafter-oligopeptides were attached to poly(L-lactide-*co*-thiol-lactide) using by a thiol-ene reaction to form polylactide-*graft*-oligopeptides.



Scheme 2. Grafting from strategy *versus* grafting to strategy for the synthesis of polylactide-*graft*-oligopeptides (involving both enzymatic synthesis and thiol-ene reactions that use AIBN as the radical initiator).

To further investigate the importance of the design of the grafter (i.e. the importance of having an alanine end group instead of an ester group) enzymatic synthesis and MD simulations of

commercially available ethyl 6-heptenoate (Figure 1, *right*) were also conducted. The results were compared to the synthesized grafter (ethyl hept-6-enoylalaninate, Figure 1 *left*).



Grafter ethyl 6-heptenoate

Figure 1. Molecular structure of ethyl hept-6-enoylalaninate (*grafter*) and ethyl 6-heptenoate.

Synthesis of the grafter

The grafter was successfully synthesized from a two-step reaction. 6-Heptenoyl chloride was prepared from 6-heptenoic acid using $SOCl_2$ as a chlorinating agent. The acid chloride was then added to a solution of alanine ethyl ester in dry CH_2Cl_2 (under N_2) and NEt_3 was used to initiate the reaction. The product was characterized by ^{1}H-, ^{13}C-, COSY and HSQC-NMR (the spectra are shown in Figure 2 and in Figure S1-S3).

The structure was confirmed by comparing the ^{1}H-NMR spectra of 6-heptenoic acid and 6-heptenoyl chloride. Because of the changes in the end groups (from a carboxylic acid to an acyl chloride), the triplet peak of the CH_2 in the alpha position (labeled "g" in figure 2) shifted from 2.39 ppm to 2.92 ppm with respect to the carbonyl carbon. In the second step, in which the chloride was replaced by an amine group, the triplet peak shifted upfield to 2.24 ppm since the amine was less electronegative than the chloride atom.

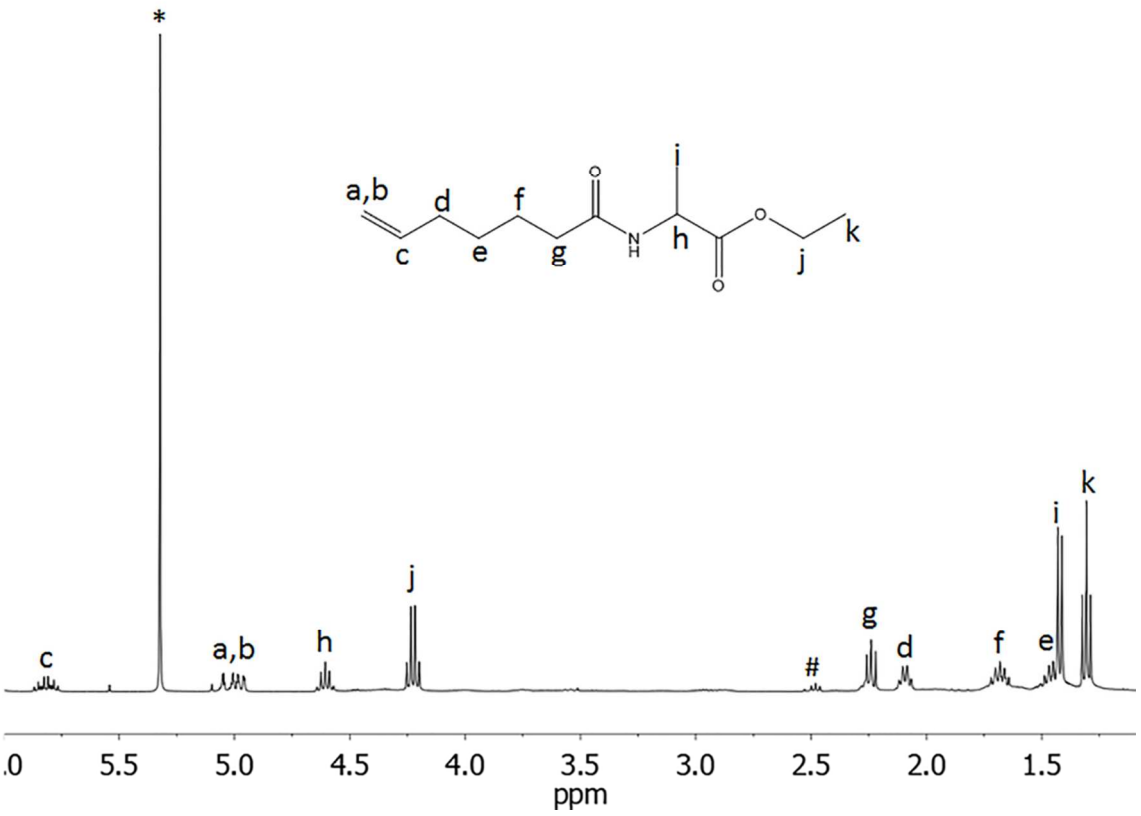


Figure 2. ^{1}H-NMR of ethyl hept-6-enoylalaninate (grafter) in $CDCl_3$ at 25 ºC. (*) indicates the solvent used in the synthesis (dichloromethane) and (#) corresponds to the residues of 6-heptenoic acid.

The grafting from strategy

The grafter was successfully attached to poly(L-lactide-*co*-thiol-lactide) via a thiol-ene click reaction, and the structure of the grafted copolymer was confirmed using ^{1}H-NMR and COSY-NMR (Figure 3 and Figure S4). In the ^{1}H-NMR spectrum, a peak at 3.92 ppm appeared (labeled b in figure 3). This peak was absent in the ^{1}H-NMR spectrum of the grafter while peaks from the double bond appeared at 5.88 and 5.07 ppm. The peaks originating from the double bond in the grafter were not observed in the ^{1}H-NMR of the polymer, indicating that all of it had reacted.

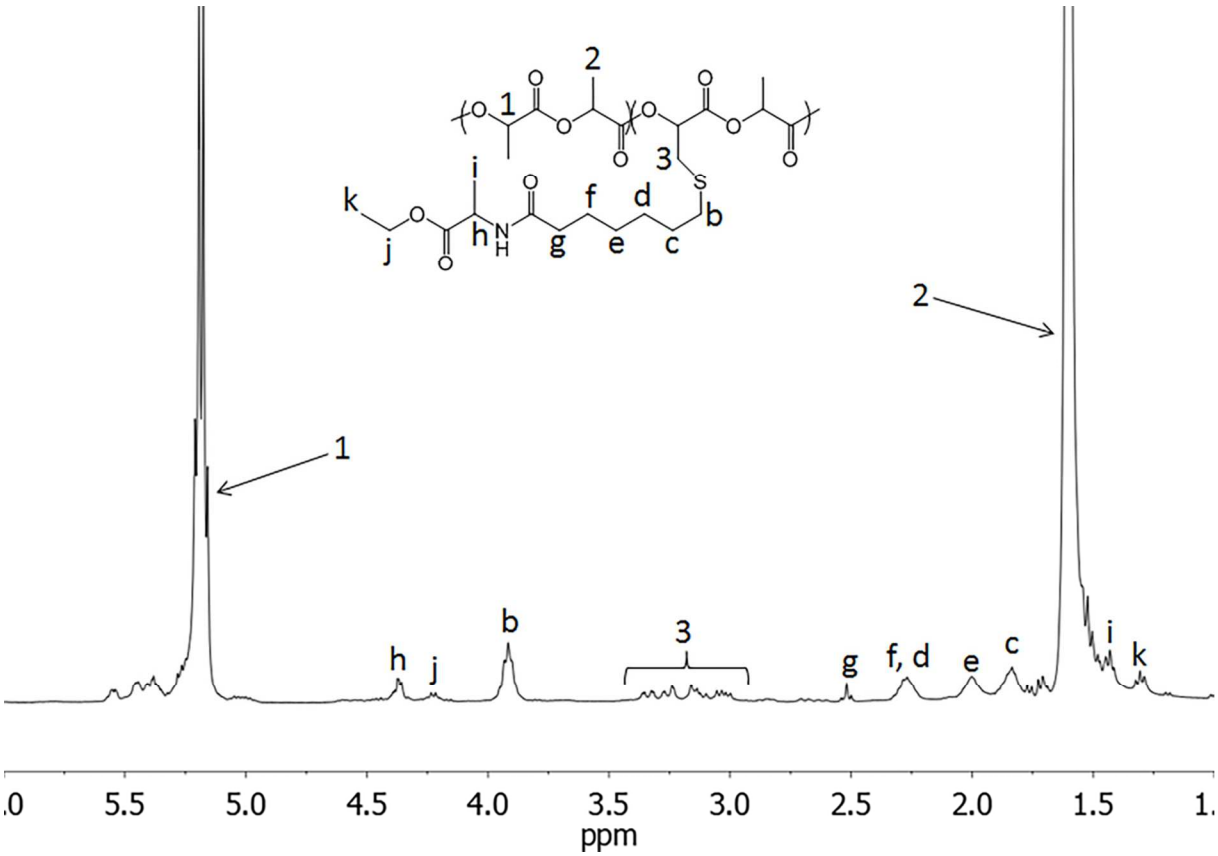


Figure 3. ^{1}H-NMR spectrum of poly(L-lactide-*co*-lactide-grafter) in $CDCl_3$ at 25 °C.

The oligopeptides were synthesized from poly(L-lactide-*co*-lactide-grafter) films using chemo-enzymatic synthesis with papain as the catalyst. The products were analyzed using ^{1}H-NMR and DMF-SEC.

When the oligopeptides were synthesized on the polylactide films, only the hydrophobic part (the copolymer and the attached grafter) of the product was visible in the ^{1}H-NMR spectrum (Figure 4, in $CDCl_3$). This was reasoned to be due amphiphilic properties of the product. In organic solvents, amphiphilic molecules form micelles, in which the hydrophilic domains are folded inward to form the core, and the hydrophobic domains form the shell. Therefore, only the hydrophobic part (i.e., the copolymer and the attached grafter) is visible in the ^{1}H-NMR spectrum, whereas the oligopeptides are not. A ^{1}H-NMR spectrum was also measured in

deuterated THF (Figure S5) since the solubility of the product was slightly higher compared to $CDCl_3$. A peak at 4.14 ppm originating from the alpha proton of the repeating unit (labeled h_n in figure S5) became visible, but the peak was weak in relation to the signals from the hydrogens in the hydrophobic regions.

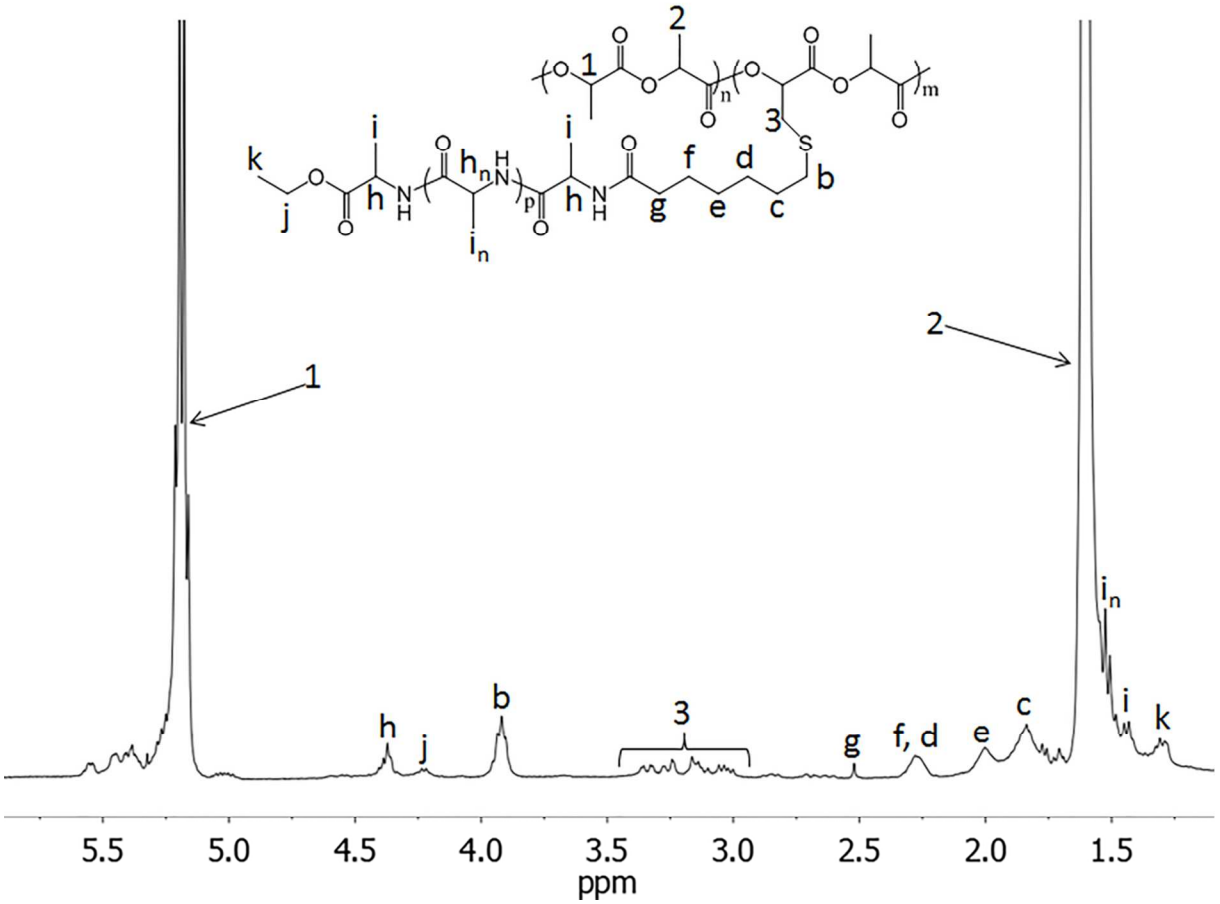


Figure 4. ^{1}H-NMR of the poly(L-lactide-*co*-lactide-grafter) films grafted with oligoalanine (polylactide-*graft*-oligoalanine) and synthesized using chemo-enzymatic synthesis in $CDCl_3$ at 25 °C. The spectrum is analogous to the other spectra in which lysine, serine or arginine were grafted instead of alanine.

To further confirm the successful grafting of the oligopeptides, the samples were also analyzed using SEC to evaluate the hydrodynamic volume change. The results summarized in Table 1 show how the molecular weight and dispersity (hence the hydrodynamic volume) increased after the enzymatic synthesis, confirming that the oligopeptides were grafted on the copolymer films.

Table 1. SEC analysis of poly(L-lactide-*co*-lactide-grafter) grafted with oligopeptides through chemo-enzymatic synthesis

Sample	***M*n [g/mol]**	**Đ**
Polylactide-*graft*-oligoalanine	14800	1.4
Polylactide-*graft*-oligolysine	13900	1.5
Polylactide-*graft*-oligoserine	13600	1.7
Polylactide-*graft*-oligoarginine	14600	1.5
Poly(L-lactide-*co*-thiol-lactide)	9200	1.1

Interestingly, white precipitates were formed during the synthesis of all samples. Although papain yields non-grated oligopeptides as a side reaction, theoretically a precipitate should only have formed during the reactions with alanine ethyl esters present, since oligoalanine is hydrophobic. Thus precipitates were presumably formed because of papain-catalyzed hydrolysis of the copolymer films. Since papain exhibits esterase activity[36], it can not only attach amino acids onto the grafter but also hydrolyze the ester bonds of the copolymer, causing it to degrade. The ^{1}H-NMR analysis of the precipitate (in comparison with that of the polylactide-*graft*-oligoserine films (Figure S6)) confirmed that degradation occurred, showing the peaks of the copolymer and repeating units of the serine and ethoxy end groups. Indeed, the DMF-SEC analysis showed that the dispersity of the copolymer increased after the enzymatic synthesis. Another drawback with this synthesis method is that the number of attached amino acids on the copolymer was difficult to control and analyze. Since the peptide part of the copolymer was not

visible in the ^{1}H-NMR spectra, this technique could not be used to determine the number of repeating units. The ongoing hydrolysis by the enzyme also cut the copolymer and the synthesized peptide chains, resulting in polymers and oligopeptides with various chain lengths.

The grafting to strategy

Peptides were first chemo-enzymatically synthesized on the grafter, forming grafter-oligopeptides (i.e., grafter-oligoalanine (grafter-oligoA), grafter-oligolysine (grafter-oligoK), grafter-oligoserine (grafter-oligoS) or grafter-oligoarginine (grafter-oligoR) depending on the amino acid ethyl ester used), which in the next step, were attached to poly(L-lactide-co-thiol-lactide) via a thiol-ene click reaction. The synthesized oligopeptides from the grafter were characterized using ^{1}H-NMR. The molecular weights, number of repeating units (DP) and monomer conversion were determined and are reported in Table 2. The spectra and calculations are presented in the supporting information (Figure S7-S10).

Table 2. Molecular weight, monomer conversion, DP and theoretical molecular weight of the grafter-oligopeptides after 18 h in a 1 M PBS buffer at 40 °C

grafter-oligopeptide	M_n [a] [g/mol]	Conversion [a] [%]	DP [a]	$M_{(theo)}$ [g/mol]
grafter-oligoA	582 ± 123	95 ± 3	5 ± 2	369
grafter-oligoK	910 ± 74	76 ± 6	5 ± 1	483
grafter-oligoS	546 ± 100	69 ± 12	4 ± 1	401
grafter-oligoR	747 ± 238	79 ± 5	4 ± 2	539

[a] Analyzed using ^{1}H-NMR in triplicate. Samples were dissolved in deuterium oxide at 25ºC.

The synthesis of the homo-oligomers of A-Et and K-Et, using papain as an enzymatic catalyst, has been described in earlier research[14,15]. The number average molecular weights (M_n) of the grafter-oligopeptides were all less than 1000 g/mol. Compared to the theoretical molecular weight ($Mn_{(theo)}$), M_n was higher. This was because not all of the grafter-oligopeptide was

catalyzed by the enzyme. The The DP of the grafter-oligoA was lower than the value reported in earlier research by K. Numata *et al*., in which homo-oligomers of nine repeating units were obtained at pH 7. This result could be explained by the fact that homo-oligopeptides of alanine longer than 4-5 repeating units are hydrophobic and precipitate from solution. The DP of grafter-oligoK corresponded to our earlier research in which homo-oligomers of lysine with a DP of four were obtained at pH 7 after 18 h.[37] In contrast, precipitates (other than unreacted grafter) were not formed in the synthesis of grafter-oligoK, -oligoS and -oligoR. They have hydrophilic side chains and therefore remain soluble in the buffer solution. Still, the number of repeating units was similar to that of grafter-oligoA. This can be explained by the fact that the peptides were exposed to the enzyme in the solution that performed not only aminolysis, linking more amino acids to the peptide, but also chain hydrolysis after a threshold DP was reached (see discussion above). The same phenomenon has been described in earlier research, in which peptides have been synthesized by proteases in aqueous buffer solutions[15,22,38].

The monomer conversions of the amino acid ethyl esters after a reaction time of 18 h were all above 60 %. Consistent with our earlier research, the A-Et conversion was almost complete (ca 95 %) while K-Et had a conversion of approximatively 76 %. Shown in earlier research[15,20], papain has a substrate specificity for amino acids with hydrophobic side chains. Therefore, the conversion of A-Et was higher than the conversion of K-Et. Similar to lysine, both serine and arginine have hydrophilic side chains. The hydroxyl groups on serine and positive charges on arginine affect the hydrophilicity of the amino acid, which explains why they also had a lower monomer conversion compared to A-Et.

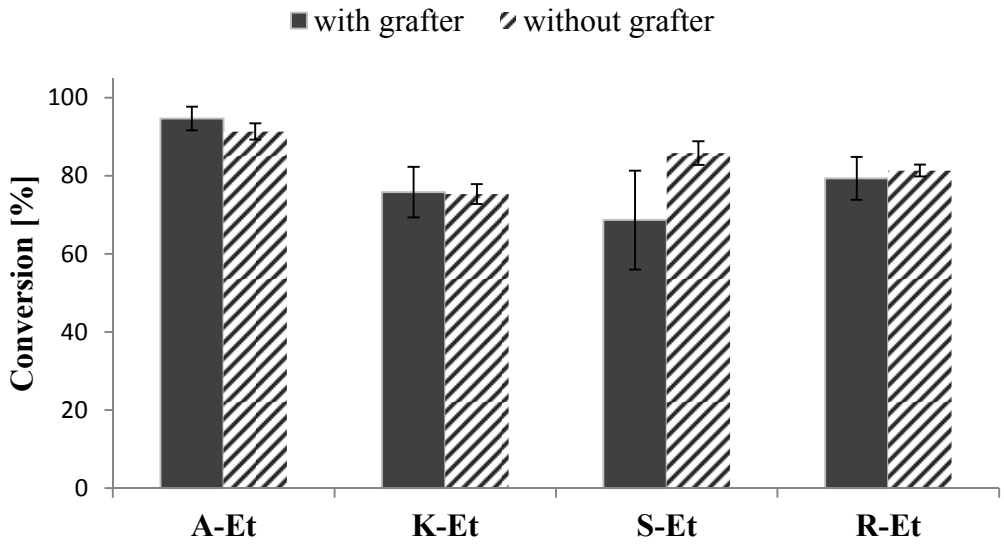


Figure 5. Monomer conversion of alanine ethyl ester (A-Et), lysine ethyl ester (K-Et), serine ethyl ester (S-Et) and arginine ethyl ester (R-Et) with and without the grafter present in the solution.

The influence of the grafter on the enzyme activity was also evaluated with respect to the inhibition of papain, which would result in decreased peptide syntheses rates. Accordingly, the conversion of the amino acid ethyl esters was analyzed with and without the addition of the grafter (figure 5). The results indicate that the presence of the grafter did not significantly affect the activity of the enzyme.

The formed grafter-oligopeptides were then attached to poly(L-lactide-*co*-thiol-lactide) using thiol-ene click chemistry with AIBN as the radical initiator. The molecular structure of the copolymer grafted with grafter-oligopeptides was analyzed using ^{1}H-NMR (Figure 6).

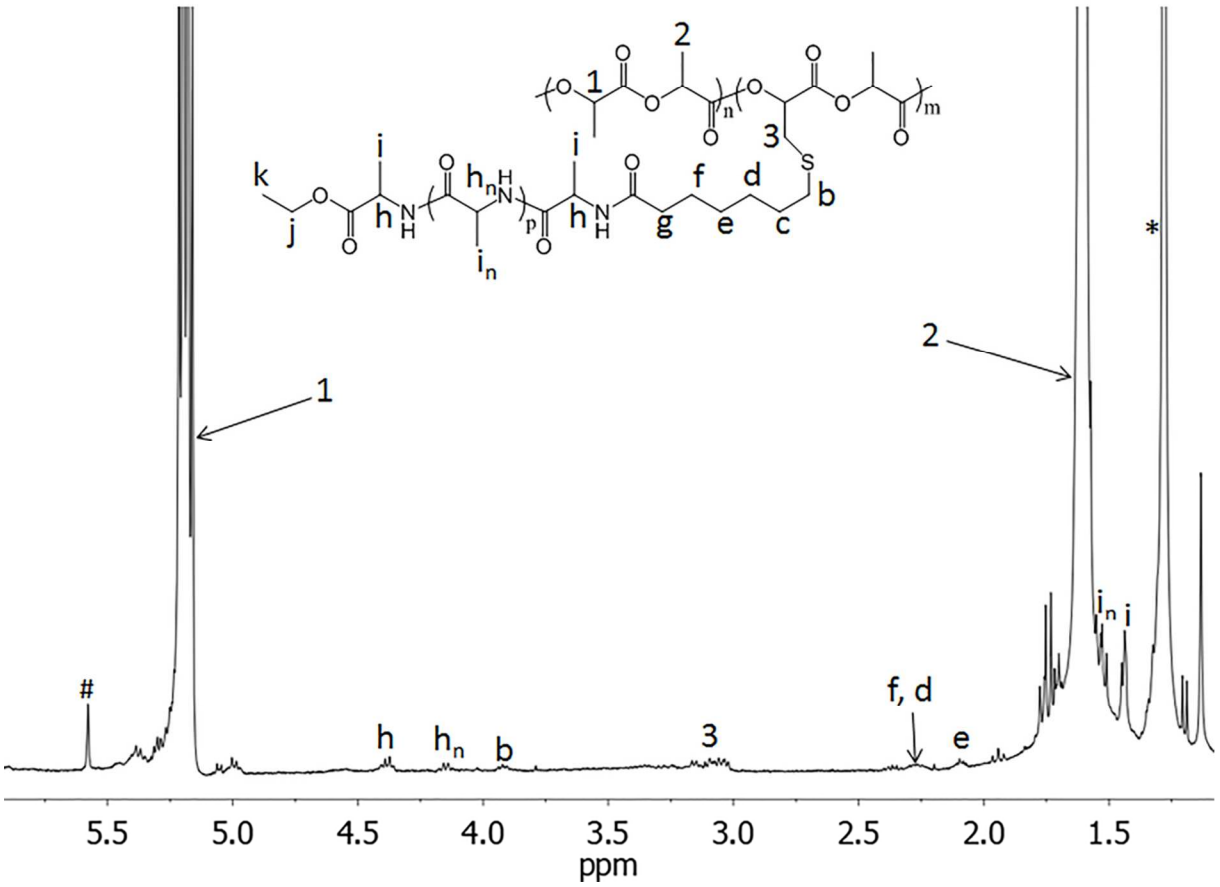


Figure 6. ^{1}H-NMR spectrum of poly(L-lactide-*co*-thiol-lactide) grafted with grafter-oligoA in $CDCl_3$, (25 °C). (*) is the solvent residue (HDO) present in the sample, and (#) is the C-H proton from the residual trityl moieties in the sample.

A peak at 3.92 ppm (labeled b in figure 6) appeared when the grafter-oligopeptide became attached to the copolymer. Similar to the polylactide-graft-oligopeptide synthesized from "the grafting from strategy", the product became amphiphilic when the grafter-oligopeptides were attached to the poly(lactide-co-thiol-lactide). Therefore, the hydrophobic portions (i.e., the polylactide main chain, which forms the shell of the micelle) are clearly seen in the spectrum, while the hydrophilic portions (i.e., the grafter-oligopeptide core) only appear as small peaks. The peak at 4.14 ppm (h_n), which originates from the alpha proton of the repeating units of the amino acids, is visible in this spectrum just like it was for the copolymer films functionalized

with peptides. Peaks from the ethoxy end groups of the peptide chain were not observed (labeled j and k). This was expected since the intensity of the peak originating from the alpha protons (h_n) is greater than the intensity of the peaks originating from the ethoxy end group.

Comparing the ^{1}H-NMR of the different oligopeptides is difficult since the peptide chains only partly dissolved in chloroform. The difficulties in dissolving polypeptides are a common problem because of the hydrogen bonding and conformational restrictions of the peptide chain. Peptides bearing charged side chains (such as lysine and arginine) also display a pH-dependent solubility and can aggregate with oppositely charged complexes.[39]

The ^{1}H-NMR spectra of the poly(L-lactide-*co*-thiol-lactide) functionalized with grafter-oligoA, grafter-oligoK, grafter-oligoS and grafter-oligoR were compared (Figure 7). All spectra confirm the attachment of oligopeptides, as indicated by the peaks at 3.92 ppm (labeled b in the spectra). Like the spectra in Figures 3, 4 and 6, this signal originates from the CH_2 connected to the thiol of the copolymer. Also present in all spectra are the peaks at 4.37 ppm (labeled h) and the peaks at 2.25 ppm (labeled f and d) originating from the protons in the grafter that are hydrophobic enough to be visible in the spectra. The alpha proton of the repeating unit of the amino acid (h_n at 4.15 ppm) is only barely visible. The signal is stronger in the spectrum in which oligoalanine is attached, but for oligolysine, oligoserine and oligoarginine - which are more hydrophilic - the intensity of the H_n peaks is very low. In addition, due to the amphiphilic properties of the polylactide-*graft*-oligopeptides, ^{1}H-NMR spectra of the samples analyzed in D_2O were also obtained (figure 8). The spectra confirm the amphiphilic properties of the product, where now peaks corresponding to the oligopeptides are shown. It should also be noted that all of the grafter-oligopeptides were not attached. Small peaks originating from the C=C protons can be seen especially in the spectrum where polylactide was grafted with oligoserine. However, the

exact amount of the unreacted grafter was not possible to determine since the signal to noise of the peaks was too low.

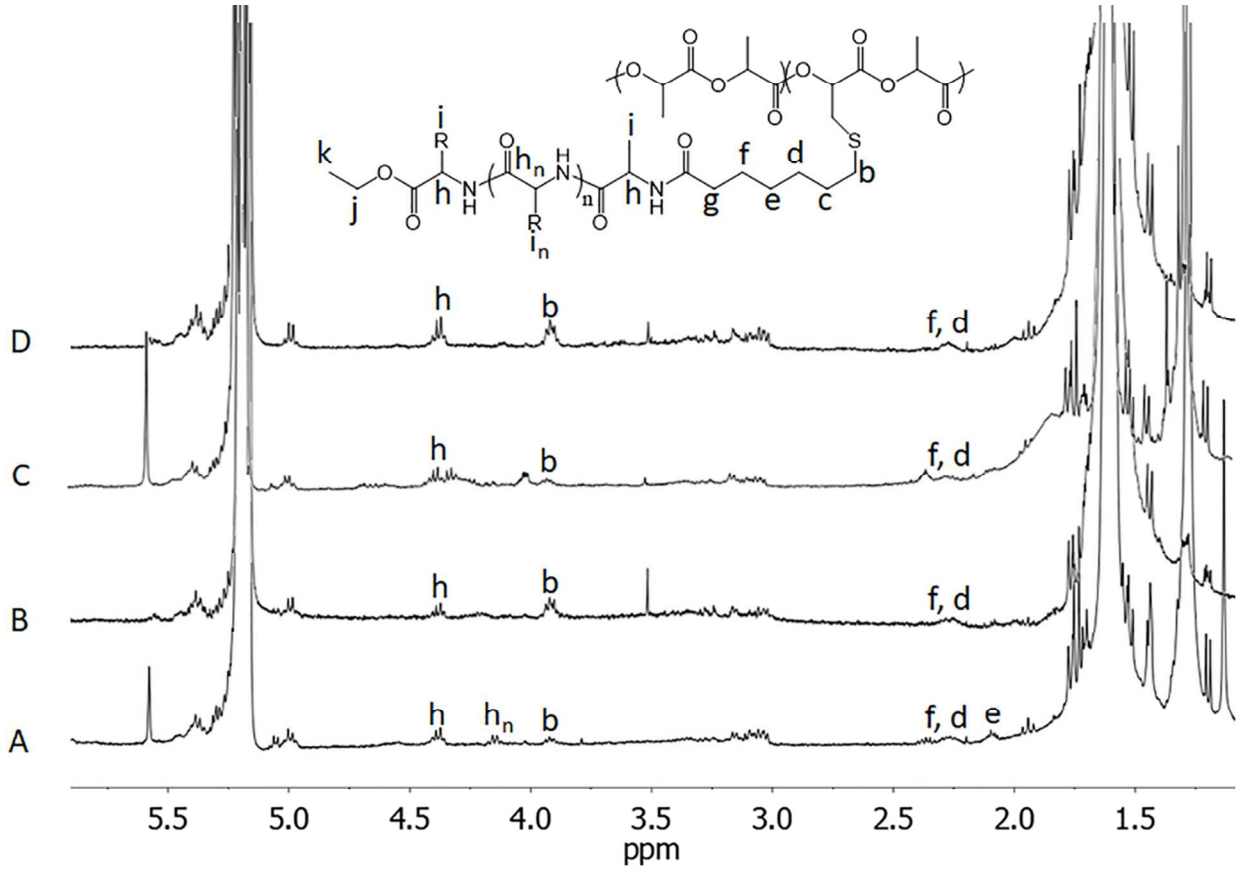


Figure 7. ^{1}H-NMR of the poly(L-lactide-*co*-thiol-lactide) grafted with A) grafter-oligoA, B) grafter-oligoK, C) grafter-oligoS and D) grafter-oligoR in $CDCl_3$ at 25 °C.

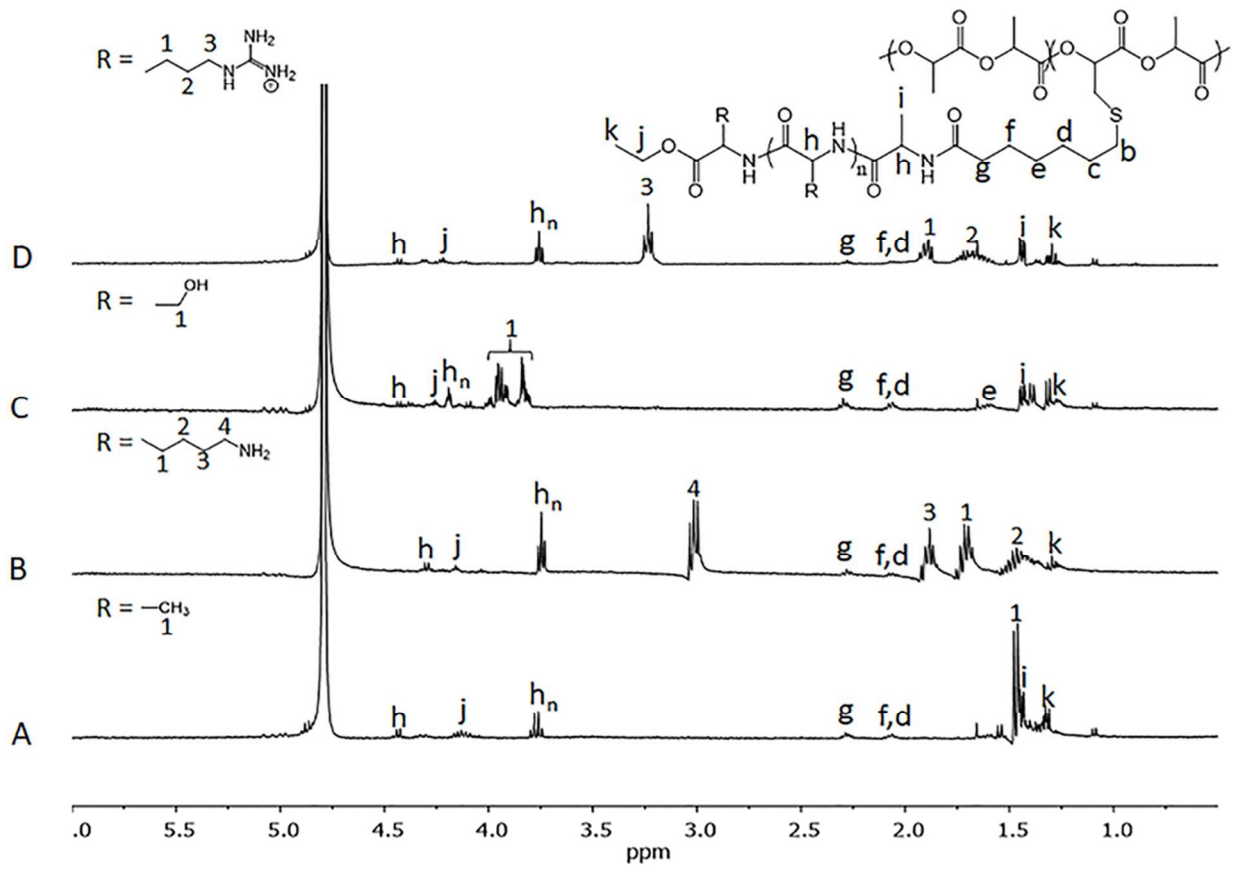


Figure 8. ^{1}H-NMR of the poly(L-lactide-*co*-thiol-lactide) grafted with A) grafter-oligoA, B) grafter-oligoK, C) grafter-oligoS and D) grafter-oligoR in D_2O at 25 □C.

To further confirm that the grafter-oligopeptides were successfully attached, the copolymers of poly(L-lactide-*co*-thiol-lactide) grafted with grafter-oligoK and grafter-oligoR were analyzed using EDS-SEM. The atomic percent (At %) of the nitrogen in the copolymers were measured. From this analysis, the amount of nitrogen was 6 % with respect to the content of oxygen, carbon and sulfur for the films containing oligoarginine, and 2 % for the films containing oligolysine. This is reasonable since arginine has three nitrogens in its side chain, while lysine only has one. An EDS-SEM image of the poly(L-lactide-*co*-thiol-lactide) grafted with grafter-oligoR film is shown in Figure S11.

To analyze the polylactide grafted with oligopeptides and the changing surface properties of the product with the different amino acids, contact angle measurements were also conducted. The contact angle of poly(L-lactide-*co*-thiol-lactide) was analyzed as a reference (Table 3).

Table 3. The contact angles of poly(L-lactide-*co*-thiol-lactide) and poly(L-lactide-*co*-thiol-lactide) grafted with grafter-oligoA, grafter-oligoK, grafter-oligoS and grafter-oligoR

Sample	Contact angle [°]	Hydrophobic/Hydrophilic
Polylactide-*graft*-oligoalanine	78.7 ± 2.7	Hydrophilic
Polylactide-*graft*-oligolysine	66.2 ± 2.6	Hydrophilic
Polylactide-*graft*-oligoserine	95.4 ± 5.6	Hydrophobic
Polylactide-*graft*-oligoarginine	73.5 ± 2.2	Hydrophilic
Poly(L-lactide-*co*-thiol-lactide)	94.6 ± 4.3	Hydrophobic

As expected, poly(L-lactide-*co*-thiol-lactide) was hydrophobic with a contact angle of 94.6°. When the copolymer was grafted with oligoalanine, oligolysine, or oligoarginine the contact angle decreased, and the surface became more hydrophilic. This is consistent with amine groups on the surface, which decreased the hydrophobicity of the copolymer. Since lysine and arginine have hydrophilic side chains, the corresponding copolymers became more hydrophilic compared to alanine, which has a hydrophobic side chain. However, when oligoserine was grafted to the copolymer, the contact angle increased, and the surface became more hydrophobic than the reference. This was not expected since serine has a hydrophilic side chain bearing a hydroxyl group and should therefore have a lower contact angle than poly(L-lactide-*co*-thiol-lactide). A possible reason could be that the attachment of oligoserine was not complete. The amount of grafted oligoserine on the PLA might be too low to observe the expected surface change in hydrophilicity.

Molecular dynamics simulations

The proposed reaction mechanism for papain-catalyzed chemo-enzymatic synthesis using the grafters and L-alanine ethyl ester (A-Et) as the substrate is shown in Figure 9. The reaction is initiated by a nucleophilic attack on the grafter carbonyl carbon by cysteine 25 (C25) assisted by histidine 159 (H159) acting as a base. The negative charge that develops on the carbonyl oxygen is stabilized by an oxyanion hole composed of the backbone amides of C25 and glutamine 19 (Q19). During the second step (i.e. deacylation), the nucleophilic amino acid ethyl ester (exemplified by alanine ethyl ester) enters the active site and attacks the acylated papain. In the first tetrahedral intermediate thus formed (TI_1), the lone pair (n) of the reacting nitrogen will be oriented towards the protonated H159 (Figure 8). For the mechanism to proceed, the lone pair sitting on the reacting nitrogen needs to be oriented antiperiplanar to the C-N bond to achieve a favorable n-σ* orbital overlap[40]. To sit in a productive conformation (i.e., $\mathbf{TI_2}$), an inversion of the lone pair from $\mathbf{TI_1}$ (or rotation of the reacting C-N bond) is required. Recently, it has been shown that hydrogen bond formation, facilitating the high-energy transition state for nitrogen inversion ($\mathbf{TS_{inv}}$), is a universal strategy displayed by amidases, regardless of protein fold or composition of catalytic dyads/triads[41-42]. For papain, the backbone carbonyl oxygen of aspartate 158 (D158) provides this stabilizing interaction by acting as a hydrogen bond acceptor in the $\mathbf{TS_{inv}}$. The description of the ability of the flexible protein backbone to remain in place to afford this key interaction remains elusive.

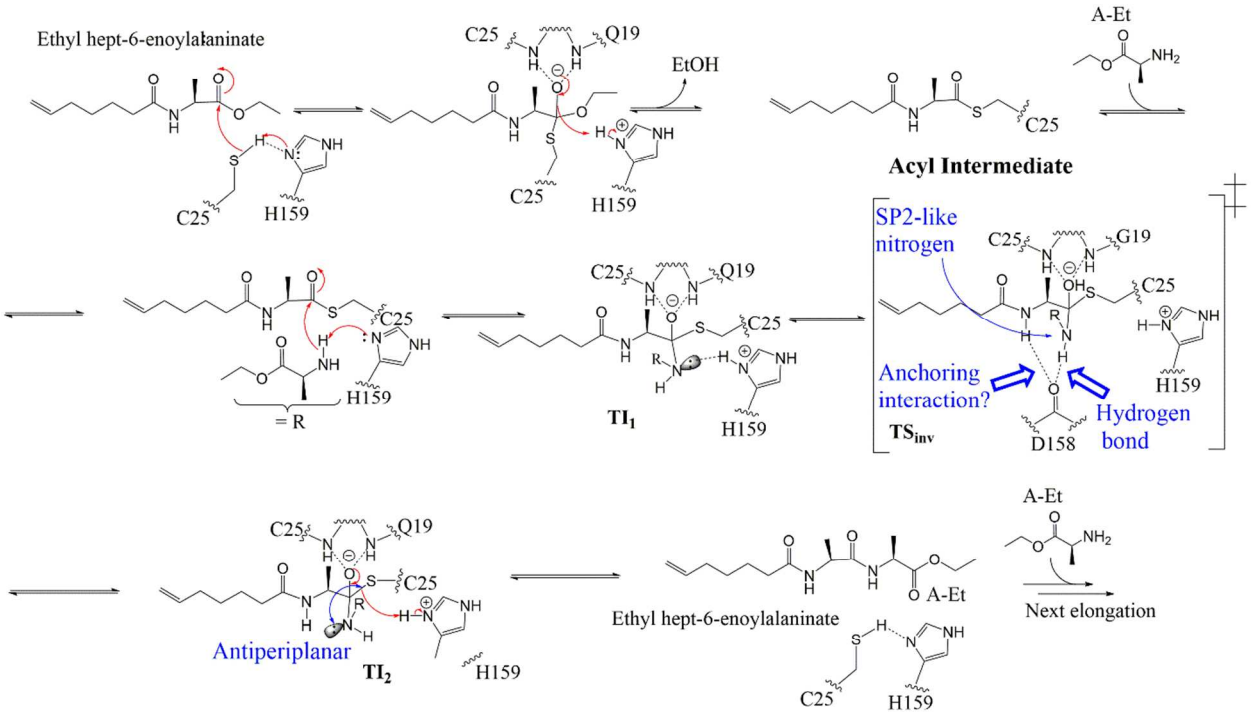


Figure 9. Reaction mechanism for papain-catalyzed peptide synthesis with grafter and A-Et. Not all transition states are shown for clarity.

To evaluate the impact of the grafters and the amino acid monomers on conversion, MD simulations were conducted on the respective tetrahedral intermediates representing the high-energy transition state structures.[41,42] For the MD trajectories, the key hydrogen bond interaction between the reacting NH-group and the backbone carbonyl of D158 was analyzed.

The MD simulations indicated that for ethyl-hept-6-enoylalaninate, the hydrogen bond acceptor, D158, is kept in place by an additional "anchoring" H-bond interaction provided by the substrate (ethyl-hept-6-enoylalaninate) P1 NH-group (Table 4, Figures 9 and 10). This enables formation of the H-bond with the reacting nitrogen of the incoming nucleophilic amino acid monomer, thus facilitating nitrogen inversion by lowering the energy of the transition state, resulting in higher conversion.

Table 4. Results from the MD simulations of the tetrahedral intermediates. For reference, conversion data are given

Grafter	Monomer	Average Distance (Å) R-NH[a] …O = D158	Average Angle (Degrees) R-N-H[a] …O = D158	H-bond Probability	Anchoring Probability	Conversion [%]
Ethyl hept-6-enoylalaninate	A-Et	2.76	163	0.10	0.81	94.6 ± 3
Ethyl hept-6-enoylalaninate	S-Et	2.72	163	0.25	0.83	75.8 ± 6.5
Ethyl hept-6-enoylalaninate	K-Et	2.76	163	0.16	0.79	68.7 ± 12
Ethyl hept-6-enoylalaninate	R-Et	2.99	160	0.12	0.70	79.3 ± 5.5
Ethyl 6-heptenoate	A-Et	3.53	153	0.0010	- [b]	~0

[a] R represents the nucleophilic amino acid monomer.

[b] Formation is not possible due to the absence of the P1 nitrogen in the ethyl 6-heptenoate backbone.

Consistent with the conversion results of the ethyl hept-6-enoylalaninate and the amino acids from the experimental part, the results from the simulations show a similar H-bond probability of 0.10-0.25 for all the amino acids in the peptide synthesis.

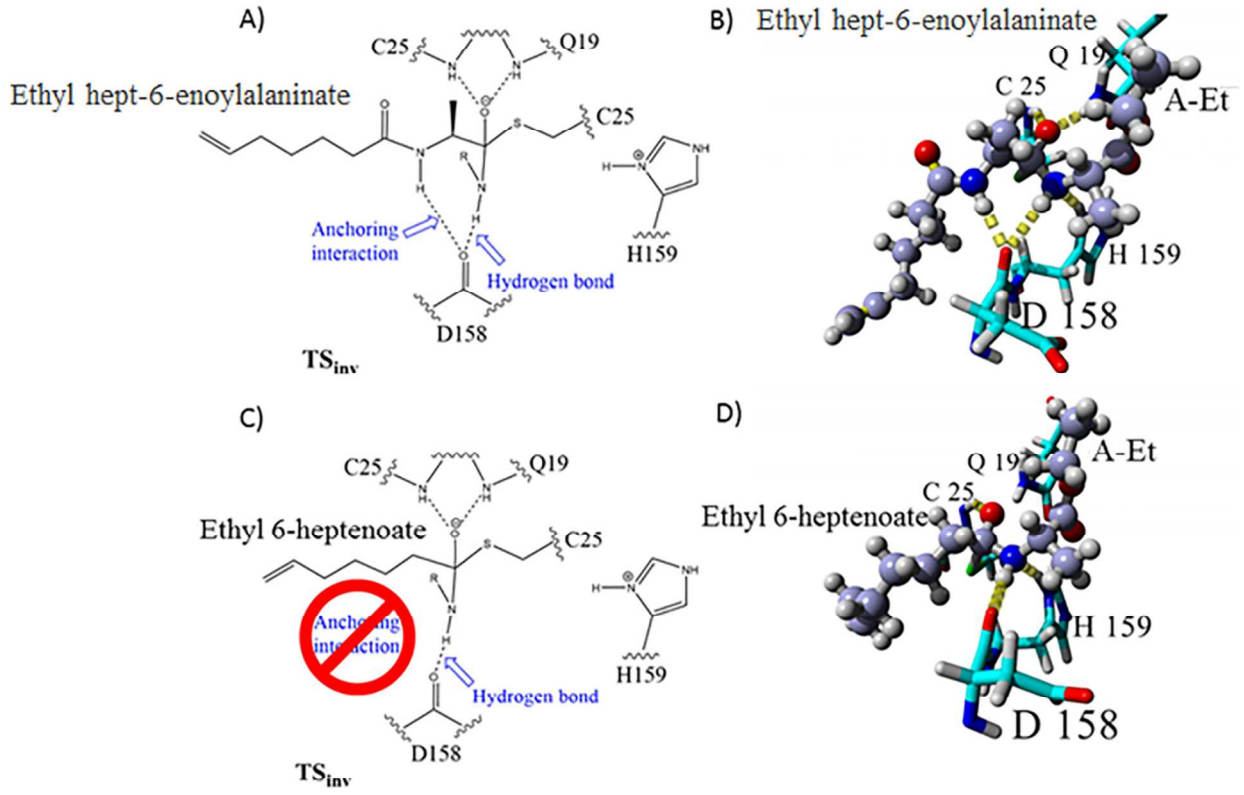


Figure 10. MD snapshots of tetrahedral intermediates representing the transition state of inversion (TS_{inv}) of the grafters (ethyl hept-6-enoylalaninate and ethyl 6-heptenoate) as acyl donors and with the amino acid monomer A-Et as nucleofile. A) TS_{inv} for the ethyl hept-6-enoylalaninate and A-Et. B) MD snapshot of the tetrahedral intermediate of ethyl hept-6-enoylalaninate and A-Et. C) TS_{inv} for ethyl 6-heptenoate lacking a P1 amide group and A-Et. D) MD snapshot of the tetrahedral intermediate of ethyl 6-heptenoate and A-Et. Note that the shown hydrogen bond between the reacting NH-group and D158 displays a 100-fold reduced relative probability (table 4).

To experimentally elucidate the importance of the proposed anchoring interaction in TS_{inv}, another synthon (ethyl 6-heptenoate), lacking the means to form this interaction, was

investigated. The α-methyl group in the grafter is not required *per se,* as papain can catalyze the conversion of peptides with glycine in the P1 position.[43]

A reaction with ethyl 6-heptenoate, papain and A-Et was run using the same conditions as those used for the chemo-enzymatic synthesis with the grafter. The results from the ^{1}H-NMR analysis showed that only free oligoalanine was present in solution and that none of the oligoalanine was bonded to the ethyl 6-heptenoate. These findings were supported by the MD simulations, where the average distance and angle between the reacting NH-group of the amino acid ethyl ester and the backbone carbonyl of D158 significantly increased (Table 4). This was associated with a concomitant 100-fold reduction in the H-bond probability in the TS_{inv} compared to that of the ethyl hept-6-enoylalaninate and A-Et (Table 4), corroborating the importance of the proposed anchoring interaction (Figure 10).

Conclusions

Chemo-enzymatic synthesis of oligopeptides from a polylactide chain was successfully initiated by a designed and synthesized grafter (ethyl hept-6-enoylalaninate). The molecular structure and performance of the grafter was analyzed using ^{1}H-NMR and MD simulations. The results showed that the design of the grafter is essential for efficient enzymatic synthesis. Specifically, the MD simulations revealed that the hydrogen bond acceptor (i.e. backbone carbonyl of D158) in papain was held in place by an anchoring interaction. This interaction, provided by the P1 backbone NH-group of the grafter, enables the formation of the key mechanistic hydrogen bond between the reacting NH-group of the incoming nucleophilic amino acid and the D158 backbone in papain. When ethyl 6-heptenoate, which lacks the P1 NH-group, was used instead of the ethyl hept-6-enoylalanineate, the favorable spatial arrangement of D158 was obstructed. This prevented stabilization of the TS for nitrogen inversion with concomitant loss of biocatalysis.

The oligopeptides were successfully grafted onto a polylactide chain using two different synthetic routes; the grafting from strategy, and the grafting to strategy. Both methods were successful and the structures of the products were confirmed by ^{1}H-NMR. The grafting to strategy was preferred since the molecular weight and number of repeating amino acids were easier to control.

ASSOCIATED CONTENT

Supporting Information

Figure S1: ^{13}C-NMR of ethyl hept-6-enoylalaninate (grafter) in $CDCl_3$ at 25 ºC;

Figure S2: COSY-NMR of ethyl hept-6-enoylalaninate (grafter) in $CDCl_3$ at 25 ºC;

Figure S3: HSQC-NMR of ethyl hept-6-enoylalaninate (grafter) in $CDCl_3$ at 25 ºC;

Figure S4: COSY-NMR of poly(L-lactide-co-lactide-grafter) in $CDCl_3$ at 25 ºC ;

Figure S5: ^{1}H-NMR of polylactide-*graft*-oligoalanine in d_6-THF at 25 ºC. The peak labeled * is solvent and # is the C-H proton from the removed trithyl group still left in the sample.

Figure S6: ^{1}H-NMR of white precipitated formed during the synthesis of polylactide-graft-oligoalanine (the grafting from strategy) in $CDCl_3$ at 25 ºC

Figure S7: ^{1}H-NMR of grafter-oligoA in D_2O at 25 ºC

Figure S8: ^{1}H-NMR of grafter-oligoK in D_2O at 25 ºC

Figure S9: ^{1}H-NMR of grafter-oligoS in D_2O at 25 ºC

Figure S10: ^{1}H-NMR of grafter-oligoR in D_2O at 25 ºC

FigureS11: EDS-SEM image of polylactide-graft-oligoarginine. The nitrogen distribution is shown in the image as yellow dots.

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