Innovative platform technologies for stabilization and controlled release of proteins from polymer depots
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Incomplete protein release from hydrophilic polylactide-PEG-b-poly(ε-caprolactone) implants

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Abstract

In this study, novel hydrophilic multiblock copolymers composed of semi-crystalline poly(ε-caprolactone) [PCL] blocks and amorphous blocks consisting of poly(D,L-lactide) (PDLA) and poly(ethylene glycol) (PEG) [PDLA-PEG] were synthesized. The block ratio of these [PDLA-PEG]-b-[PCL] multiblock copolymers was varied and the degradation of implants prepared of these polymers by hot melt extrusion (HME) was compared with implants prepared of [PCL-PEG]-b-[PCL], a copolymer which has been described previously [1]. It was shown that the initial degradation rate of the [PDLA-PEG]-b-[PCL] multiblock copolymers increased with increasing the content of amorphous [PDLA-PEG] block and that the degradation rate of these multiblock copolymers was faster than that of the [PCL-PEG]-b-[PCL] multiblock copolymers due to rapid degradation of the [PDLA-PEG] block.

Furthermore, the release of the model proteins lysozyme and bovine serum albumin from polymer implants prepared by HME was studied. It was found that the protein release from [PDLA-PEG]-b-[PCL] copolymers was incomplete, in contrast to [PCL-PEG]-b-[PCL] copolymers that showed slow and continuous release. We hypothesize that the incomplete release is explained by an irreversible interaction between the proteins and polymer degradation products or by entrapment of the protein in the hydrophobic and non-swellable polymer matrix that was left after degradation and loss of the hydrophilic [PDLA-PEG] blocks from the degrading polymer
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1. Introduction

In recent years, there has been a growing interest in the application of polymers as matrices for controlled release drug delivery systems. More specifically, from the mid 1970’s, more and more research has been done on biodegradable polymers as an alternative to non-biodegradable polymers [2]. Contrary to non-biodegradable polymers that require surgical removal, biodegradable polymers are susceptible to degradation in the body into small molecules either by chemical or enzymatic hydrolysis or both [3]. Among this class of polymers, polyesters offer many beneficial properties when used as drug delivery depots, such as biocompatibility, low toxicity, and degradation into monomers that can enter metabolic pathways or be excreted via the kidney. In order to be used as a drug delivery depot, they should also possess adequate mechanical, chemical, physical and thermal properties [4]. Moreover, the polymer should provide a suitable microenvironment for the encapsulated drug and avoid any significant changes in pH due to accumulation of acidic degradation products, and they should exhibit control over the release rate. Since biodegradation can be an important factor determining the release rate, tailored biodegradation is preferable. Poly(D,L-lactide), poly(glycolide), poly(ε-caprolactone), and especially copolymers thereof, either or not in combination with poly(ethylene glycol) (PEG), have been extensively studied as sustained release drug delivery matrices [5–8].

Despite the increased use of biodegradable polymers for various applications, there are still some problems that hinder the widespread application in drug depot systems. Those problems are associated with the complexity and interactions of factors that play a role in the production of implants and the drug release from the polymer depots. Some of these factors are related to the physicochemical characteristics of the drug, including drug instability, hydrophilicity or hydrophobicity and molecular weight, while others are related to the physicochemical and degradation behavior of the polymer, such as polymer composition, polymer crystallinity, polymer hydrophilicity / hydrophobicity, molecular weight, molecular weight distribution, geometry of the implant, processing conditions, site of application [9–11].

Poly(ε-caprolactone) (PCL) is a highly biocompatible polymer with a low melting temperature and high permeability to low molecular weight drugs [12]. However, PCL degrades slowly, both in-vitro and in-vivo, which in combination with its highly hydrophobic nature and crystalline structure limits its application in drug delivery depots [13,14]. Copolymerization of ε-caprolactone with other monomers provides a means to adjust its physical-chemical characteristics and degradation rate.

Block copolymers composed of hydrophobic poly(DL-Lactide) or poly(ε-caprolactone) (PCL) in combination with hydrophilic PEG [15–17] offer the
possibility of varying the hydrophilicity and swelling degree of the polymer and allow modulation of drug release and degradation kinetics of the copolymer. More hydrophilic polymers generally provide better compatibility with protein drugs. Furthermore, an increased swelling degree allows for more continuous diffusion-based release, preventing the biphasic release profile that is typically encountered for poly(lactide-co-glycolide)-based protein delivery matrices.

In a previous study, we introduced phase separated multiblock copolymers composed of semi-crystalline PCL blocks and amorphous blocks consisting of PCL and PEG [PCL-PEG]-b-[PCL] [1]. We described that by changing the block ratio of these multiblock copolymers, thereby changing their hydrophilicity / hydrophobicity balance and swelling degree, both protein release and polymer degradation rate can be tailored. However, degradation of these [PCL-PEG]-b-[PCL] based multiblock copolymers containing 22.5 or 37.5 wt% PEG was relatively slow, while at 57.5 wt% PEG the multiblock copolymer degraded fast due to dissolution of hydrophilic polymer chains. The slow degradation was attributed to the presence of relatively large fractions of slow degrading PCL in both the amorphous and the crystalline blocks.

The aim of the present study was to replace the amorphous [PCL-PEG] blocks by an amorphous blocks composed of poly(D,L-lactide) and PEG ([PDLA-PEG]) as to obtain faster degrading copolymer systems. The effect of the [PDLA-PEG] / [PCL] block ratio (and thus the PEG content), on polymer degradation kinetics and on the release kinetics of the model proteins lysozyme and bovine serum albumin from polymeric implants prepared by HME was studied and compared with that of the previously reported [PCL-PEG]-b-[PCL] multiblock copolymer.

2. Materials and methods

2.1. Materials

Lyophilized lysozyme (Lys) (from chicken egg white ~14 kDa), lyophilized albumin from bovine serum (BSA), (protein >96 %, ~66 kDa), stannous octoate, dimethylformamide (DMF), Micrococcus lysodeikticus, acetonitrile (HPLC gradient grade), ethyl acetate, dimethylsulfoxide (DMSO), dichloromethane, sodium azide, sodium chloride, disodium hydrogen phosphate, potassium dihydrogen phosphate, trifluoroacetic acid, hydroxylamine hydrochloride, sodium dodecyl sulphate (SDS), urea and dithiotretiol (DTT), were purchased from Sigma. ε-Caprolactone,
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D,L-lactide, PEG (Mw 1000 g/mol), 1,4-butanediol and 1,4-dioxane were obtained from Acros, Geel, Belgium. 1,4-Butanediisocyanate was purchased from Bayer. Deuterated chloroform and lithium bromide were obtained from Fisher and PEG standards were purchased from Fluka.

2.2. Polymer synthesis

PCL and poly(D,L-lactide)-PEG\textsubscript{1000}-b-poly(D,L-lactide) [PDLA-PEG\textsubscript{1000}] prepolymers were synthesized by standard stannous octoate catalyzed ring-opening polymerization, as described previously [18]. ε-Caprolactone was dried over CaH\textsubscript{2} and distilled under reduced pressure in an atmosphere of dry nitrogen. D,L-lactide was dried overnight at 50 °C under vacuum. PEG with a molecular weight of 1000 g/mol (PEG\textsubscript{1000}) was dried overnight at 90 °C under vacuum. 1,4-Butanediol and 1,4-butanediisocianate were distilled under reduced pressure. The purity of the distilled ε-caprolactone, 1,4-butanediol and 1,4-butanediisocyanate was confirmed by \textsuperscript{1}H NMR (CDCl\textsubscript{3}). PCL prepolymer with a target molecular weight of 4000 g/mol was prepared by introducing 241 g (2.11 mol) of anhydrous ε-caprolactone into a three-necked bottle under an atmosphere of dry nitrogen and adding of 5.6 g (62.03 mmol) of anhydrous 1,4-butanediol to initiate the ring-opening polymerization. Stannous octoate was used as a catalyst at a catalyst / monomer molar ratio of 8.40 \times 10\textsuperscript{-5}/1. The mixture was magnetically stirred for 70 h at 140 °C and subsequently cooled to room temperature.

[PDLA-PEG\textsubscript{1000}] prepolymer with a target molecular weight of 2000 g/mol, was synthesized in a similar way using 150 g (1.04 mol) of D,L-lactide, 150 g (149.21) mmol of PEG\textsubscript{1000} and molar catalyst / monomer ratio of 2.72 \times 10\textsuperscript{-4}/1. The mixture was magnetically stirred for 10 days at 140 °C and subsequently cooled to room temperature.

[PCL] and [PDLA-PEG\textsubscript{1000}] prepolymers were then chain-extended with 1,4-butanediisocyanate to prepare \textsubscript{x/y} [PDLA-PEG\textsubscript{1000}]-y[PCL] multiblock copolymer where \textsubscript{x/y} is the [PDLA-PEG\textsubscript{1000}]/[PCL] weight ratio, being 10/90, 20/80, 30/70 or 50/50 (Table 1). [PCL] and [PDLA-PEG\textsubscript{1000}] were introduced into a three-necked bottle under an atmosphere of dry nitrogen. Dry 1,4-dioxane (distilled over sodium wire) was added and the mixture was heated to 80 °C to obtain a solution of the prepolymers with a concentration of 30 wt%. 1,4-Butanediisocyanate was added and the reaction mixture was mechanically stirred for 20 h. After cooling to room temperature, the reaction mixture was transferred into a tray, frozen and vacuum-dried at 30 °C to remove 1,4-dioxane. Synthesis and characterization of 30[PCL-PEG\textsubscript{1500}]-70[PCL] (previously abbreviated as 30CP15C20-C40) has been described elsewhere [18].
### Table 1. In weights of pre-polymers used in synthesis of \(x\text{[PDLA-PEG}_{1000}]\)-\(y\text{[PCL]}\) multiblock copolymers.

<table>
<thead>
<tr>
<th></th>
<th>PDLA-PEG(_{1000}) prepolymer</th>
<th>PCL prepolymer</th>
<th>PDLA-PEG(_{1000}) prepolymer</th>
<th>PCL prepolymer</th>
<th>BDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>In weights</td>
<td>wt%</td>
<td>wt%</td>
<td>In weights</td>
<td>In weights</td>
<td>In weights</td>
</tr>
<tr>
<td>10\text{[PDLA-PEG}_{1000}]-90\text{[PCL]}</td>
<td>10</td>
<td>90</td>
<td>10.28 g (5.09 mmol)</td>
<td>88.01 g (21.68 mmol)</td>
<td>3.22 g (22.95 mmol)</td>
</tr>
<tr>
<td>20\text{[PDLA-PEG}_{1000}]-80\text{[PCL]}</td>
<td>20</td>
<td>80</td>
<td>19.84 g (9.82 mmol)</td>
<td>77.96 g (19.59 mmol)</td>
<td>3.51 g (25.05 mmol)</td>
</tr>
<tr>
<td>30\text{[PDLA-PEG}_{1000}]-70\text{[PCL]}</td>
<td>30</td>
<td>70</td>
<td>28.30 g (14.15 mmol)</td>
<td>63.57 g (15.97 mmol)</td>
<td>3.79 g (27.07 mmol)</td>
</tr>
<tr>
<td>50\text{[PDLA-PEG}_{1000}]-50\text{[PCL]}</td>
<td>50</td>
<td>50</td>
<td>48.42 g (23.97 mmol)</td>
<td>47.16 g (11.85 mmol)</td>
<td>5.08 g (36.24 mmol)</td>
</tr>
</tbody>
</table>

2.3. Polymer characterization

\(^1\text{H}\) NMR was used to determine monomer conversion, number average molecular weight (Mn) and overall chemical composition of the polymer after synthesis and during degradation. \(^1\text{H}\) NMR was performed on a VXR Unity Plus NMR Machine (Varian, California, USA) operating at 300 MHz. The \(d_1\) waiting time was set to 20 s, and the number of scans was 16-32. \(^1\text{H}\) NMR samples were prepared by dissolving 10 mg of polymer in 1 mL of deuterated chloroform (CDCl\(_3\)), and the spectrum was determined from 0 - 8 ppm using CHCl\(_3\) present as trace element in CDCl\(_3\) as reference.

Monomer conversion was calculated from peaks originating from the polymer and the monomer. For [PCL], monomer conversion was calculated from the peaks of the \(-\text{O-CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{C(O)}-\) methylene groups of [PCL] and monomer \(\varepsilon\)-caprolactone at \(\delta\) 2.2-2.5 and \(\delta\) 2.65, respectively. For [PDLA-PEG\(_{1000}\)], monomer conversion was calculated from the peaks of the \(-\text{O-CH(H)}\text{(CH)}_3\text{C(O)}-\) methylene groups of PDL and monomer D,L-lactide at \(\delta\) 5.1-5.4 and \(\delta\) 5.0-5.1, respectively. The experimental number average molecular weight of the [PCL] prepolymer was determined by \(^1\text{H}\) NMR using the peaks of the methylene end groups of PCL at \(\delta\) 3.6-3.7 and the \(-\text{O-CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{C(O)}-\) methylene group of PCL at \(\delta\) 2.2-2.5. The experimental Mn of the [PDLA-PEG\(_{1000}\)] prepolymer was determined by the peak of the PDLA methylene groups \(-\text{O-CH(H)}\text{(CH)}_3\text{C(O)}-\) at \(\delta\) 5.1-5.4 and the peaks of the PEG methylene groups \(-\text{CH}_2\text{CH}_2\text{O}-\) at \(\delta\) 3.6-3.7.

\(^1\text{H}\) NMR was also used to verify the overall \(\varepsilon\)-caprolactone/PEG (CL/PEG) and D,L-lactide/PEG (LA/PEG) monomer ratio of the multiblock copolymers. CL/PEG molar ratio was calculated from the \(\text{O-CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{C(O)}-\) methylene
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group of PCL and ε-caprolactone at δ 2.2 - 2.5 and δ 2.65, respectively, and the –CH₂CH₂-O methylene groups of PEG at δ 3.6 - 3.7. PDLA/PEG molar ratio was calculated from the -O-CH(CH₃)C(O)- methyne groups of PDLA and D,L-lactide monomer at δ 5.1 - 5.4 and δ 5.0 - 5.1, respectively, and the –CH₂CH₂-O methylene groups of PEG at δ 3.6 - 3.7. The intrinsic viscosity of the polymer dissolved in chloroform was determined by measuring the dynamic viscosity at three different polymer concentrations at a temperature of 25 °C using an Ubbelohde viscometer (DIN, type 0C Schott Geräte supplied with a Schott AVS-450 Viscometer equipped with a water bath).

The residual 1,4-dioxane content of the multiblock copolymer was determined using a GC-FID headspace method. Measurements were performed on a GC-FID Combi Sampler supplied with an Agilent Column, DB-624/30 m/0.53mm. Samples were prepared in DMSO. 1,4-Dioxane content was determined using 1,4-dioxane calibration standards. The apparent molecular weight of the multiblock copolymers was determined using size exclusion chromatography (SEC-HPLC, Waters, Breeze, USA). The polymers were dissolved in DMF (0.01 g/mL). PEG standards having molecular weights of 1 - 218 kg/mol were prepared likewise. Samples and PEG standards were injected (50 μL) onto the SEC column (Thermo Fisher, Column 1: Plgel 5 μm 500 Å, column 2: Plgel 5 μm 500 Å, column 3: Plgel 5 μm 104 Å, eluent: DMF with 0.1 M LiBr, flow: 1 mL/min). Polymers were detected by refractive index. The apparent Mn and apparent weight average molecular weight (Mw) were calculated with the aid of the PEG standards calibration curve.

Modulated differential scanning calorimetry (DSC) was used to determine the thermal behavior of the multiblock copolymers and was performed using Q2000 differential scanning calorimeter (TA instruments, Ghent, Belgium). About 5 - 10 mg of dry material was heated from -85 °C to 100 °C at a rate of 2 °C / min with amplitude of 0.318 °C over a 60 s period. During the measurement, the sample cell was purged with nitrogen. The reversed heat flow was used for determination of the glass transition temperature (Tg, midpoint), while the total heat flow was used for determination of the melting temperature (maximum of endothermic peak, Tm) and the heat of fusion, which was calculated from the surface area of the melting endotherm. Temperature and heat flow were calibrated using indium.

2.4. Hot melt extrusion

For the preparation of implants HME was performed using a HAAKE MiniLab Rheomex CTW5 co-rotating twin-screw extruder (Thermo-Electron). Implant formulations were prepared from x[PDL-PEG₆₀₀₀]-y[PCL] multiblock copolymers with x/y being 10/90, 20/80, 30/70 or 50/50 (w/w), loaded with no proteins.
(polymer-only implants) or 10 wt% of Lys or BSA, and from 30[PCL-PEG\textsubscript{1500}]-70[PCL] loaded with 10 wt% Lys, as listed in a Table 2. Since protein particle size may influence the release kinetics from polymer matrices [18,19], BSA particles were milled in a stainless steel container with an aid of stainless steel beads, using a tumbling mixer (Turbula T2X, WA Bachofen AG, Switzerland) until the volume-averaged particle size was approximately 18 µm, which was similar to the volume-averaged particle size of Lys, as determined by laser diffraction (Sympatec GMBH, Clausthal-Zellerfeld, Germany). Milled BSA or Lys powder were then physically mixed with polymer powder and fed into the preheated barrel of the extruder. Extrusion was performed at 50 - 55 °C using a screw speed of 10 - 20 rpm and a torque of 4 - 7 Nm. A cylindrical die of 0.5 mm was used, resulting in strands with a diameter of 0.35 mm, as measured with an in-line laser. Polymer-only implants, used for the degradation study were extruded similarly, without further additives. Polymer strands were cut into pieces of 2 cm x 0.35 mm (for the degradation study) and 1 cm x 0.35 mm (for the in-vitro release study) and stored at -20 °C prior to use.

Table 2. Protein-loaded and polymer-only implant formulations prepared by HME.

<table>
<thead>
<tr>
<th>Formulation name and polymer grade</th>
<th>Protein</th>
<th>PEG content in multiblock copolymer (wt%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lys/10[PDLA-PEG\textsubscript{1000}]-90[PCL]</td>
<td>Lys</td>
<td>5</td>
</tr>
<tr>
<td>Lys/20[PDLA-PEG\textsubscript{1000}]-80[PCL]</td>
<td>Lys</td>
<td>10</td>
</tr>
<tr>
<td>Lys/30[PDLA-PEG\textsubscript{1000}]-70[PCL]</td>
<td>Lys</td>
<td>15</td>
</tr>
<tr>
<td>Lys/50[PDLA-PEG\textsubscript{1000}]-50[PCL]</td>
<td>Lys</td>
<td>25</td>
</tr>
<tr>
<td>BSA/10[PDLA-PEG\textsubscript{1000}]-90[PCL]</td>
<td>BSA</td>
<td>5</td>
</tr>
<tr>
<td>BSA/20[PDLA-PEG\textsubscript{1000}]-80[PCL]</td>
<td>BSA</td>
<td>10</td>
</tr>
<tr>
<td>BSA/30[PDLA-PEG\textsubscript{1000}]-70[PCL]</td>
<td>BSA</td>
<td>15</td>
</tr>
<tr>
<td>BSA/50[PDLA-PEG\textsubscript{1000}]-50[PCL]</td>
<td>BSA</td>
<td>25</td>
</tr>
<tr>
<td>Lys/30[PCL-PEG\textsubscript{1500}]-70[PCL]</td>
<td>Lys</td>
<td>22.5</td>
</tr>
<tr>
<td>10[PDLA-PEG\textsubscript{1000}]-90[PCL]</td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td>20[PDLA-PEG\textsubscript{1000}]-80[PCL]</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>30[PDLA-PEG\textsubscript{1000}]-70[PCL]</td>
<td>-</td>
<td>15</td>
</tr>
<tr>
<td>50[PDLA-PEG\textsubscript{1000}]-50[PCL]</td>
<td>-</td>
<td>25</td>
</tr>
<tr>
<td>30[PCL-PEG\textsubscript{1500}]-70[PCL]</td>
<td>-</td>
<td>22.5</td>
</tr>
</tbody>
</table>
2. 5. **In-vitro polymer degradation**

The degradation of polymer-only implants was evaluated during 180 days. About 130 ± 5 mg (n=3) of polymeric implants were placed in plastic vials and 25 ml of phosphate buffer (PBS) (100 mM, pH 7.4 ± 0.2, 9.1 mM NaCl, 0.02 wt% NaN₃) was added to each vial. The vials were then incubated in an oven at 37°C. pH was regularly measured and adjusted to pH 7.4 ± 0.2 using 1 M NaOH. At various time intervals, samples were removed from the buffer and washed with ultra-pure water over a 0.45 μm filter to remove the buffer salts. Adherent water from the implants was removed with a tissue. Wet mass was determined (\(m_{\text{wet},t}\)) after which the samples were dried in a desiccator for 15 h and then in a vacuum oven (30 °C, pressure < 0.01 mbar) for 24 h and weighted again (\(m_{\text{dry},t}\)). Water content and mass loss were calculated using Eq. (1) and Eq. (2).

\[
\text{Water content (\%) } = 100 \times \frac{m_{\text{wet},t} - m_{\text{dry},t}}{m_{\text{wet},t}} \quad (1)
\]

\[
\text{Mass loss (\%) } = 100 \times \frac{m_{\text{dry},0} - m_{\text{dry},t}}{m_{\text{dry},0}} \quad (2)
\]

Where \(m_{\text{dry},0}\) is the mass of the dry sample at day 0, \(m_{\text{dry},t}\) is the mass of the dry sample at time t and \(m_{\text{wet},t}\) is the mass of the wet sample at time t.

One-way ANOVA was used to determine whether the differences in water content and mass loss between the samples during degradation were significant; the difference was considered to be significant when p < 0.05. Additionally, the samples were analyzed using \(^1\)H-NMR, SEC and DSC, as described above.

2. 6. **In-vitro protein release and quantification**

The \textit{in-vitro} protein release was evaluated in 100 mM PBS (pH 7.4 ± 0.2, 9.1 mM NaCl, 0.02 wt% NaN₃). Approximately 30 mg of polymer implants (n = 3) were incubated in 1.3 ml of the release buffer and test tubes were placed vertically in a shaking water bath, under mild agitation, thermostated at 37 °C for a total of 180 days. At different time intervals, 1.1 ml of aliquots were removed for HPLC analysis and refreshed. The cumulative amount of released protein within 4h was considered as burst release.

Lys concentrations were measured with Dionex Ultimate 3000 HPLC (Thermo Scientific, Sunnyvale, CA, USA), equipped with C18 ProZap LC/MS reversed phase column (Grace Davidson, Deerfield, IL, USA) (20 x 4.6 mm, 1.5 μm). Chromatographs were obtained using an UV detector at 280 nm. Gradient system consisted of 0.1 vol% trifluoroacetic acid in acetonitrile (A) and 0.1 vol% trifluoroacetic acid in ultrapure water (B). The solvent flow rate was 1 mL/min.
and the gradient was applied for 6 min, using the following scheme: 0 min-1 min: A/B = 3/7 (v/v); 1–3 min: A/B = 6/4 (v/v); 3.01-6 min: A/B = 3/7 (v/v). The retention time of Lys was 1.19 min. Data were analyzed with Chromeleon software.

BSA concentrations were measured by an Acquity UPLC system (Waters, Milford, MA, USA) using a BEH300 C4 reversed phase column (50 x 2.1 mm, 1.7 μm). Chromatographs were obtained using a UV detector at 280 nm. The mobile phase consisted of 0.1 vol% trifluoroacetic acid in acetonitrile (A) and 0.1 vol% trifluoroacetic acid in ultrapure water. The solvent flow rate was 0.7 ml/min and gradient was applied using following program: 0–1 min A/B= 1/9 (v/v); 1–3 min A/B 4.5 / 5.5 (v/v); 3-4 min; A/B 9/1; 4–5min A/B 1/9 (v/v). The retention time of BSA was 3.5 min.

2.7. Content uniformity and structural integrity of proteins

To determine the actual protein content and structural integrity of proteins after extrusion, proteins were extracted from the implants. Around 10 - 15 mg of samples (n = 3) randomly taken during the extrusion run were weighted and 1.5 ml of ethyl acetate was added to each sample until the polymer was fully dissolved [18]. Samples were centrifuged (Microcentrifuge SIGMA 1-14, Shropshire, United Kingdom) and the supernatant containing the dissolved polymer was removed. The procedure was repeated three times where after the remaining protein pellet was dried in a desiccator overnight and then dissolved in 1 ml of a 100 mM phosphate buffer, pH 7.4 and analyzed as described in section 2.6. Control experiments using physical mixtures of polymer and protein showed that the extraction procedure did not affect the biological activity of the protein and that by the extraction procedure the proteins were completely recovered. In addition, the extraction was repeated during the in-vitro release experiments. After 30 and 105 days of the in-vitro protein release, polymer implants were taken from the medium, dried in a desiccator for 24h until constant mass and extraction was performed as described above.

The biological activity of Lys was measured by a turbidimetric assay as described by Gorin et al. [20], adopted for a plate reader [18]. Since no biological assay is available for BSA, the structural integrity of this protein was assessed semi-quantitatively, by the determination of additional peaks in the chromatograms assessed using the RP-HPLC method.
2. 8. Protein-polymer interaction

As will be described in the results section, in most cases the polymer implants showed no complete release of the incorporated proteins during the release experiment. This incomplete release may be due to protein aggregation in the implant or an irreversible interaction with the polymer. To elucidate this, the implants were subjected to various assays. To determine whether the protein was aggregated and/or non-covalently bonded to polymer, urea (6 M) was used as a denaturant due to its capability to dissociate non-covalent bonds. Sodium hydroxide (1 M) was added to provide alkaline hydrolysis of ester bonds. To investigate whether thioester bonds and disulfide bridges were formed, dithiothreitol was added (DTT, 0.01 M). Further, to selectively determine if only thioester (and not disulfide) bonds were formed, hydroxylamine hydrochloride was used (0.2 M, pH 7.4) [21,22]. Finally, to investigate whether only aggregation occurred, or if any molecules were non-covalently bound, 5 mM SDS was added to the protein-loaded implants [23].

2. 9. Scanning Electron Microscopy

The surface morphology of the implants after 180 days of degradation was investigated with SEM (JEOL, JSM 6301-F or JCM-5000 Neoscope Microscope, JEOL, Japan). Implants were attached to a double-sided carbon tape and coated with gold.

3. Results

3. 1. Polymer synthesis and characterization

The results of the characterization of x[PDLA-PEG\textsubscript{1000}]-y[PCL] multiblock copolymers by IV, \textsuperscript{1}H NMR, SEC and DSC are summarized in Table 3. Similar IV values were obtained for the various multiblock copolymers, ranging from 0.61 - 0.73 dL/g. As expected from the similar IV values, SEC indicated that the apparent molecular weights of the various multiblock copolymers were also similar as the \textit{Mn} and \textit{Mw} values ranged from 15.4 - 21.3 kg/mol and 31 - 40 kg/mol, respectively. The polydispersity index, defined as the ratio \textit{Mw} / \textit{Mn}, ranged from 1.76 - 2.20.

From the \textsuperscript{1}H NMR spectra it was calculated that polymerization of \textepsilon-caprolactone resulted in the formation of [PCL] with a Mn of 3490 g/mol, which
was reasonably close to the theoretical value of 3980 g/mol, as calculated from in weight values and monomer conversion. The Mn of [PDLA-PEG\textsubscript{1000}] was 1950 g/mol, which was also close to the theoretical value of 1950 g/mol, as determined from in weights and monomer conversion.

Furthermore, \textsuperscript{1}H NMR was used to verify the overall D,L-lactate/polyethylene glycol (LA/PEG) and \(\varepsilon\)-caprolactate / polyethylene glycol (CL/PEG) molar ratios of the multiblock copolymer. The overall LA/PEG molar ratio of the \(x\)[PDLA-PEG\textsubscript{1000}]-\(y\)[PCL] multiblock copolymers ranged from 14.5 - 17.4 mol / mol, which was close to the theoretical LA / PEG molar ratio from in-weights (13.9 - 14.2 mol / mol). CL/PEG molar ratio of the \(x\)[PDLA-PEG\textsubscript{1000}]-\(y\)[PCL] multiblock copolymers varied with varying PCL block content. CL/PEG molar ratios ranged from 16.8 - 135, as determined by \textsuperscript{1}H NMR and were found close to the theoretical CL/PEG molar ratio as calculated from in weights (15.1 - 148) (Table 1).

DSC confirmed the phase-separated morphology of the multiblock copolymers, showing a \(T_g\) between -54 and -39 °C originating from the amorphous phase and a \(T_m\) between 49 and 55 °C originating from the crystalline PCL phase. The \(T_g\) can be ascribed to the homogeneous mixture of amorphous PEG and PCL [24]. The melting enthalpy (\(\Delta H\)) of the multiblock copolymers was in the range of 61 - 74 J/g.

1,4- Dioxane was well removed from all \(x\)[PDLA-PEG\textsubscript{1000}]-\(y\)[PCL] multiblock copolymers, as its residual content was below the quantification limit of the GC-FID method (< 200 ppm).

The characterization of 30[PCL-PEG\textsubscript{1500}]-70[PCL] has been previously described [18].
Incomplete protein release from hydrophilic poly(D,L-lactide-PEG)-b-poly(ε-caprolactone) implants

Table 3: IV, $^1$H NMR, SEC and DSC results of x[PDL-PEG$_{1000}$]- y[PCL] multiblock copolymers

<table>
<thead>
<tr>
<th>Multiblock copolymers composition</th>
<th>IV (dL/g)</th>
<th>LA / PEG molar ratio</th>
<th>CL / PEG molar ratio</th>
<th>SEC (kg/mol)</th>
<th>Tg$^a$ (°C)</th>
<th>Tm$^a$ (°C)</th>
<th>$\Delta H$$^{a,b}$ (J/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>in-weights</td>
<td>$^1$H NMR</td>
<td>in-weights</td>
<td>$^1$H NMR</td>
<td>Mn</td>
<td>Mw</td>
</tr>
<tr>
<td>10[PDLA-PEG$_{1000}$]- 90[PCL]</td>
<td>0.67</td>
<td>14.2</td>
<td>16.2</td>
<td>148.2</td>
<td>134.9</td>
<td>15.4</td>
<td>30.9</td>
</tr>
<tr>
<td>20[PDLA-PEG$_{1000}$]- 80[PCL]</td>
<td>0.71</td>
<td>14.2</td>
<td>17.4</td>
<td>64.4</td>
<td>68.0</td>
<td>21.3</td>
<td>37.5</td>
</tr>
<tr>
<td>30[PDLA-PEG$_{1000}$]- 70[PCL]</td>
<td>0.73</td>
<td>13.9</td>
<td>15.8</td>
<td>38.1</td>
<td>38.5</td>
<td>18.0</td>
<td>39.6</td>
</tr>
<tr>
<td>50[PDLA-PEG$_{1000}$]- 50[PCL]</td>
<td>0.61</td>
<td>14.2</td>
<td>14.5</td>
<td>15.1</td>
<td>16.8</td>
<td>21.3</td>
<td>40.0</td>
</tr>
</tbody>
</table>

a) Determined after HME
b) Total of the PCL fraction of the multiblock copolymers, calculated by $\Delta H$(J/g PCL) = ($\Delta H_{m\text{PCL}}$/ wt% PCL), where $H_{m\text{PCL}}$ is the melting enthalpy of the PCL block per gram of multiblock copolymer, wt%$_{\text{PCL}}$ is calculated by $^1$H-NMR
3. 2. Protein content uniformity and integrity

The amount of extracted protein of randomly collected samples of the various implants only exhibited small variations, suggesting a homogeneous distribution of the protein within the polymer matrix with an average loading of 9.08 ± 0.66 % (Lys) and 9.42 ± 0.44 % (BSA) in the x[PDLA-PEG1000]-y[PCL] polymers and 10.05 ± 0.01 % (Lys) in 30[PCL-PEG1500]-70[PCL] polymer. Both proteins preserved their structural integrity during extrusion as evidenced by the absence of additional peaks in the HPLC chromatograms. In addition, the biological assay indicated that the enzymatic activity of Lys was fully preserved after extrusion.

3. 3. In-vitro polymer degradation

3. 3. 1. Water content

The in-vitro degradation study showed substantial water uptake by the polymer implants during the first day of incubation. The water content increased with increased [PDLA-PEG1000] / [PCL] block ratio and thus increased amorphous block content of the multiblock copolymer. After the first day of incubation, equilibrium was reached and water content remained more or less constant throughout the duration of the study and was 58 ± 2.1 %, 59 ± 1.7 %, 63 ± 0.7 %, 68 ± 4.9 % for 10[PDLA-PEG1000]-90[PCL], 20[PDLA-PEG1000]-80[PCL], 30[PDLA-PEG1000]-70[PCL], and 50[PDLA-PEG1000]-50[PCL], respectively (Figure 1a). Water content of 30[PCL-PEG1500]-70[PCL] after one day of incubation was 56 ± 1.9 % and also remained constant during the entire study. The difference in water content between copolymers 10[PDLA-PEG1000]-90[PCL], 30[PDLA-PEG1000]-70[PCL] and 50[PDLA-PEG1000]-50[PCL] was significant. Furthermore, the difference in water content between 20[PDLA-PEG1000]-80[PCL] and 50[PDLA-PEG1000]-50[PCL] as well as between 30[PCL-PEG1500]-70[PCL] and 50[PDLA-PEG1000]-50[PCL] was significant.

3. 3. 2. Mass loss

During incubation the mass loss of x[PDLA-PEG1000]-y[PCL] copolymers with a block ratio x/y of 10/90 and 20/80 was slow and continuous. The copolymers with a block ratio x/y of 30/70 and 50/50 exhibited, after an initial mass loss during the first 14 days, the absence of any substantial mass loss during the 180 days period thereafter. The mass loss for 10[PDLA-PEG1000]-90[PCL] was only 5.7 ± 0.1 % after 180 days of incubation (Figure 1b). With increased [PDLA-PEG1000] / [PCL] block
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ratio, and thus with an increased percentage of the amorphous block, the percentage of mass loss increased amounting to 14.7 ± 0.7 % for 20[PDLA-PEG\textsubscript{1000}]-80[PCL], 28.9 ± 0.9 % for 30[PDLA-PEG\textsubscript{1000}]-70[PCL] and 50.6 ± 0.9 % for 50[PDLA-PEG\textsubscript{1000}]-50[PCL] after 180 days of incubation. The reference copolymer, 30[PCL-PEG\textsubscript{1500}]-70[PCL] having PCL in the amorphous block, showed only 8.4 ± 0.8 % of mass loss during 140 days. The differences in the rate of mass loss among all polymers were significant.

3. 3. 3. Decrease of molecular weight

It was found that during the first 14 days of incubation the Mn decreased faster when the [PDLA-PEG\textsubscript{1000}] / [PCL] block ratio of the multiblock copolymers was increased (Figure 1c). The Mn of 10[PDLA-PEG\textsubscript{1000}]-90[PCL] decreased around 20 % during the first 14 days, after which it decreased rapidly, resulting in a decrease of more than 64 % after 180 days. During the first 14 days, the Mn of 20[PDLA-PEG\textsubscript{1000}]-80[PCL] already decreased to 60 % of its original value, while for 30[PDLA-PEG\textsubscript{1000}]-70[PCL] and 50[PDLA-PEG\textsubscript{1000}]-50[PCL] the Mn decreased almost 70 %. After the rapid decrease of the Mn of these copolymers during the first 14 days, the rate of Mn decrease declined and was similar for x[PDLA-PEG\textsubscript{1000}]-y[PCL] copolymers with an x/y ratio of 20/80, 30/70 and 50/50, resulting in 70 - 75 % decrease in Mn after 180 days.

Contrary to multiblock copolymers containing PDLA in the amorphous block, 30[PCL-PEG\textsubscript{1500}]-70[PCL] having PCL in the amorphous block showed only 15 % decrease in Mn during 140 days of incubation [1].
Chapter 6

Figure 1. Percentage of water content (a), mass loss (b) and (c) Mn loss for multiblock copolymers during degradation: 10\([\text{PDLA-PEG}_{1000}] - 90[\text{PCL}]\) (dashed line, ♦), 20\([\text{PDLA-PEG}_{1000}] - 80[\text{PCL}]\) (solid line, ■), 30\([\text{PDLA-PEG}_{1000}] - 70[\text{PCL}]\) (solid line, ▲), 50\([\text{PDLA-PEG}_{1000}] - 50[\text{PCL}]\) (dashed line, ■) and 30\([\text{PCL-PEG}_{1500}] - 70[\text{PCL}]\) (dashed line, ×).
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3.3.4. Crystallinity of the ε-caprolactone block during degradation

The crystallinity of the ε-caprolactone block during degradation was investigated. As can be seen in Figure 2, during the initial stages of degradation, the melting enthalpy of the PCL blocks of the various [PDLA-PEG$_{1000}$]-[PCL] polymers increased from 60-70 J/g to approximately 80-90 J/g, indicating increased crystallinity of the PCL during degradation of the polymers.

![Figure 2](image)

Figure 2. The melting enthalpy of the crystalline [PCL] of the multiblock copolymers during degradation, corrected for the total PCL content, 10[PDLA-PEG$_{1000}$]-90[PCL] (♦), 20[PDLA-PEG$_{1000}$]-80[PCL] (■), 30[PDLA-PEG$_{1000}$]-70[PCL] (▲), 50[PDLA-PEG$_{1000}$]-50[PCL] (●).

3.3.5. Polymer composition

$^1$H NMR showed that the relative ε-caprolactone content of all four x[PDLA-PEG$_{1000}$]-y[PCL] multiblock copolymers increased during incubation, while the PEG and PDLA contents decreased, indicating that degradation mainly occurred in the amorphous blocks. The $^1$H NMR data for a representative polymer, 30[PDLA-PEG$_{1000}$]-70[PCL], are shown in Figure 3. Within 10 days, the lactic acid content of this polymer had decreased from its original 15 wt% to 0 %. In contrast to the multiblock copolymers containing PDLA in the amorphous block, the composition of 30[PCL-PEG$_{1500}$]-70[PCL], having PCL in the amorphous block, did not substantially change (data not shown).
Figure 4. Surface of the polymer-only implants after 180 days of incubation in PBS. (a) 10[PDLA-PEG$_{1000}$]-90[PCL]; (b) 20[PDLA-PEG$_{1000}$]-80[PCL]; (c) 30[PDLA-PEG$_{1000}$]-70[PCL]; (d) 50[PDLA-PEG$_{1000}$]-50[PCL]; (e) 30[PCL-PEG$_{1500}$]-70[PCL] (140 days of incubation).

3.4. **In-vitro protein release**

The burst release of BSA and Lys from x[PDLA-PEG$_{1000}$]-y[PCL] copolymers largely depended on the [PDLA-PEG$_{1000}$] / [PCL] block ratio (and thus on the PEG content) of the polymer (**Figure 5**). With increasing [PDLA-PEG$_{1000}$] / [PCL] block ratio, the burst release increased. For the implants with the highest amorphous block
Incomplete protein release from hydrophilic poly(D,L-lactide-PEG)-b-poly(e-caprolactone) implants

content, i.e. 50[PDLA-PEG\textsubscript{1000}]-50[PCL], all Lys and 70 % of the BSA was released within one day and no further release of BSA was observed after the burst. It was also observed that after 17 days, release of both Lys and BSA ended for all formulations. In contrast, 30[PCL-PEG\textsubscript{1500}]-70[PCL], containing PCL instead of PDLA in the hydrophilic block showed an initial burst of 20 % followed by a slow and continuous Lys release until 70 % of the protein was released after 180 days (after which the release was not further followed).

As mentioned earlier, immediately after extrusion quantitative recovery of protein from both x[PDLA-PEG\textsubscript{1000}]-y[PCL] and 30[PCL-PEG\textsubscript{1500}]-70[PCL] implants was possible after extraction of the polymer with ethyl acetate. After 35 days of incubation, however, it appeared to be impossible to dissolve all polymer and therefore complete recovery of the protein from x[PDLA-PEG\textsubscript{1000}]-y[PCL] implants could not be achieved. Contrary to x[PDLA-PEG\textsubscript{1000}]-y[PCL]-based implants where after 35 days more than 40 % of polymer could not be dissolved in ethyl acetate, 30[PCL-PEG\textsubscript{1500}]-70[PCL] had almost complete protein recovery upon extraction after 35 days. Attempts to dissolve polymer of the x[PDLA-PEG\textsubscript{1000}]-y[PCL]-based protein releasing implants in other organic solvents or solvent systems (acetone, acetonitrile, DMSO/0.05N NaOH + 0.5 % SDS, dichloromethane), which are described in literature as suitable solvents for similar polymeric systems [1,25,26] were unsuccessful as well. It was hypothesized that irreversible interaction of the proteins with polymer degradation products formed during incubation of the implants could be the reason for incomplete protein release and poor solubility of the multiblock copolymer implants in the organic solvents.

![Figure 5. In-vitro release of Lys (a) and BSA (b) from multiblock copolymers (n=3): 10[PDLA-PEG\textsubscript{1000}]-90[PCL] (♦), 20[PDLA-PEG\textsubscript{1000}]-80[PCL] (■), 30[PDLA-PEG\textsubscript{1000}]-70[PCL] (▲), 50[PDLA-PEG\textsubscript{1000}]-50[PCL] (●) and 30[PCL-PEG\textsubscript{1500}]-70[PCL] (*).](image)
3. 5. Interaction of protein and polymer

To investigate the interaction between the proteins and the degrading polymer in more detail and to elucidate the cause for incomplete release, implants that showed incomplete release were collected after 17 days from the release buffer and then incubated in several media for one day, after which the amount of released protein was determined. Samples incubated with 6 M urea released only negligible amounts of the additional protein, while no further protein release was observed upon addition of DTT, hydroxylamine HCl and SDS. However, samples incubated with 1 M NaOH (Table 4) released additional 50 % of the both incorporated BSA and Lys, which cumulated to 94 % of total protein released. Based on these findings, it can be concluded that the reason for incomplete release was not disulfide bridge formation, nor aggregation/denaturation. Furthermore, upon addition of 1 M NaOH most of the protein was recovered. The almost complete recovery of protein upon full polymer degradation with 1M NaOH implies that an irreversible/covalent linkage of proteins with degradation products of the polymers could be responsible for the incomplete release. Another possible explanation for the lack of full protein recovery could be a molecular rearrangement of the semi-crystalline blocks during degradation in which the protein became entrapped. After the hydrolysis of the ester bonds with NaOH, the protein may have been released from these semi-crystalline blocks.

Table 4. Cumulative amount of protein released after 17 days of incubation of protein-loaded 30[PDLA-PEG1000]-70[PCL] implants in PBS; Further release of protein upon addition of different reagents and incubation for 24h.

<table>
<thead>
<tr>
<th>% released</th>
<th>% released</th>
<th>% released</th>
<th>% released</th>
<th>% released</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lys</td>
<td>BSA</td>
<td>Lys</td>
<td>BSA</td>
<td>Lys</td>
</tr>
<tr>
<td>t =17 days</td>
<td>43.00</td>
<td>43.10</td>
<td>43.29</td>
<td>45.96</td>
</tr>
<tr>
<td>Addition of:</td>
<td>NaOH</td>
<td>UREA 6M</td>
<td>DTT</td>
<td>SDS</td>
</tr>
<tr>
<td>t =24h</td>
<td>93.60</td>
<td>93.63</td>
<td>46.57</td>
<td>47.40</td>
</tr>
</tbody>
</table>
Incomplete protein release from hydrophilic poly(D,L-lactide-PEG)-b-poly(e-caprolactone) implants

4. Discussion

In this study, novel, biodegradable phase-separated multiblock copolymers composed of [PDLA-PEG\textsubscript{1000}] and [PCL] at various block ratio were synthesized. It was shown that the degradation rate of phase-separated [PCL-PEG\textsubscript{1500}]-[PCL] multiblock copolymers described in [1] can be enhanced by replacing [PCL-PEG\textsubscript{1500}] by [PDLA-PEG\textsubscript{1000}] in the amorphous block. It was further shown that two model proteins, BSA and Lys, can be incorporated into [PDLA-PEG\textsubscript{1000}]-[PCL] implants by HME at relatively low temperatures (50 - 55 °C), without compromising their structural integrity.

The degradation of aliphatic esters is known to start by water penetration into the amorphous regions of the polymer bulk, often accompanied by swelling, which induces hydrolysis of the ester bonds [27]. This chemical degradation results in the formation of oligomers and monomers. Progressive degradation creates pores in the bulk microstructure through which monomers and oligomers can diffuse out, resulting in mass loss [28], [29], [30].

Our results demonstrated that the molecular weight of the copolymers decreases immediately upon contact with water. The decrease in Mn was attributed to polymer chain hydrolysis, which occurred mainly in the hydrophilic and amorphous [PDLA-PEG] block of the copolymer. The preferential degradation of the [PDLA-PEG] blocks is supported by \textsuperscript{1}H-NMR data, which showed that the content of lactic acid and PEG (and thus the content of the [PDLA-PEG] block) decreased and that the relative amount of PCL increased during degradation. Also, in line with these findings, for all x[PDLA-PEG\textsubscript{1000}]-y[PCL] copolymers the percentage mass loss did not exceed the mass percentage of the amorphous block in the copolymer.

For the x[PDLA-PEG\textsubscript{1000}]-y[PCL] multiblock copolymers with higher [PDLA-PEG\textsubscript{1000}] / [PCL] block ratio mass loss occurred immediately after 1 day of incubation, while for the copolymers with lower [PDLA-PEG\textsubscript{1000}] / [PCL] block ratio, mass loss was substantially slower. These results are in line with the finding that swelling and thus the rate of hydrolysis increased with increased amorphous block content (i.e. increased [PDLA-PEG\textsubscript{1000}] / [PCL] block ratio). Mass loss of the multiblock copolymer with [PCL-PEG\textsubscript{1500}] as the amorphous block (8.4 % in 140 days) was slow as compared to copolymer with [PDLA-PEG\textsubscript{1000}] as the amorphous block and with similar PEG content and degree of swelling (29 % in 140 days), which can be ascribed to the relatively slow degradation of [PCL-PEG\textsubscript{1500}] as compared to [PDLA-PEG\textsubscript{1000}] [31]. From the DSC data, it was clear that the crystallinity of the PCL block increases during degradation.
It has previously been described that burst release of protein is most likely due to dissolution of particles from the polymer surface followed by dissolution and liberation of the neighboring particles [18]. In this study, we observed that the burst release increased with increasing [PDLA-PEG\textsubscript{1000}] / [PCL] block ratio, which implies that for this type of copolymers, besides dissolution of the particles from or close to the surface also polymer swelling and subsequent degradation played a role in the initial release. Polymers with a [PDLA-PEG\textsubscript{1000}] / [PCL] block ratio of 30/70 and 50/50 exhibited a certain mass loss already during the burst phase, supporting that the increased burst release with increased [PDLA-PEG\textsubscript{1000}] / [PCL] block ratio may also be ascribed to liberation of the proteins together with parts of the [PDLA-PEG\textsubscript{1000}] blocks of the polymer.

As shown earlier [1] for low swellable polymers, the molecular weight of the protein is an important factor affecting the release rate. Proteins of a higher molecular weight will not be released unless polymer erosion occurs. Hence, with a high content of hydrophilic [PDLA-PEG\textsubscript{1000}] block and thus enhanced polymer swelling, the release is mainly driven by dissolution / degradation of the [PDLA-PEG\textsubscript{1000}] block leading to release of both smaller and larger proteins. In the present study, we showed that, except for Lys that was completely released from 50[PDLA-PEG\textsubscript{1000}]-50[PCL] already in the initial phase, no further protein release was observed for both Lys and BSA after the burst release for the x[PDLA-PEG\textsubscript{1000}]-y[PCL] copolymers. In addition, it appeared that after incubation for 35 days the implants were not soluble in various organic solvents in which the implants could be dissolved before incubation. These findings might be due to an irreversible interaction of the proteins with the polymer degradation products, which were still present in the implant. In contrast to the x[PDLA-PEG\textsubscript{1000}]-y[PCL] implants, the release of Lys from 30[PCL-PEG\textsubscript{1500}]-70[PCL] implant was continuous during the entire duration of the release study. Furthermore, 30[PCL-PEG\textsubscript{1500}]-70[PCL] based Lys containing implants could be dissolved almost completely in ethyl acetate after 35 days of incubation. As 30[PCL-PEG\textsubscript{1500}]-70[PCL] only moderately degrades during the period of incubation [1], the incomplete release from the [PDLA-PEG\textsubscript{1000}]-[PCL] copolymers has to be related to the degradation of these polymers.

Incomplete protein release from biodegradable polymer matrices has been observed before but little is known about the exact mechanism. Protein release can be compromised by protein adsorption to the polymer or by protein aggregation [26,32,33]. Other authors reported that incomplete peptide or protein release can be due to the chemical modification of proteins during incubation, e.g. by acylation [21,34–37]. In order to assess the nature of the interaction between protein and polymer, the implants were exposed to aqueous solution of urea, SDS, DTT, hydroxylamine HCL and NaOH after 17 days of incubation. It was shown that only
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Sodium hydroxide could liberate the protein most likely due to its ability to hydrolyze the ester bonds and completely degrade the polymer, which suggests irreversible interaction or covalent bonding between the protein and [PDLA-PEG\textsubscript{1000}] related degradation products as a cause for incomplete protein release. However, it has also been observed that after a few days of incubation, the crystallinity of PCL was substantially increased indicating molecular rearrangements of PCL of the semi-crystalline PCL blocks. Therefore, it cannot be ruled out that during these molecular rearrangements of the PCL blocks the protein was physically entrapped in these structures instead of irreversibly interacted with the polymer degradation products in the implant, which has also been shown before [38]. However, if the protein would remain physically entrapped, it should have been recovered upon the protein extraction using ethyl acetate.

In conclusion, our results show that the initial degradation rate and the overall mass loss of the multiblock copolymers can be increased by replacing slowly degrading [PCL-PEG\textsubscript{1500}] by rapidly degrading [PDLA-PEG\textsubscript{1000}] block. We have shown that copolymers composed of x[PDLA-PEG\textsubscript{1000}]-y[PCL] exhibit continuous degradation rate in the initial phase, which can be controlled by varying the [PDLA-PEG\textsubscript{1000}] / [PCL] block ratio. According to our expectations, faster degradation resulted in accelerated protein release. However, incomplete protein release was observed. We concluded that either irreversible interaction between the protein and degradation products originating from the [PDLA-PEG\textsubscript{1000}] block or the physical entrapment of the protein into the semi-crystalline hydrophobic PCL matrix compromised protein release. Contrary, protein release from the slowly degrading x[PCL-PEG\textsubscript{1500}]-y[PCL] copolymers was continuous during the entire study, implying the absence of protein-polymer interactions or protein entrapment for this type of copolymer.

Further studies towards the successful development of protein loaded polymer depot formulations should be directed towards elucidation of the exact mechanism of incomplete protein release and towards the fundamental understanding of the molecular interaction between protein and polymer degradation products.

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References


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