Innovative platform technologies for stabilization and controlled release of proteins from polymer depots
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Low temperature extruded implants based on novel hydrophilic multiblock copolymer for long-term protein delivery

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Abstract

Parenteral protein delivery requires preservation of the integrity of proteins and control over the release kinetics. In order to preserve the integrity, parenteral protein delivery formulations typically need to be processed at low temperatures. Therefore, we synthesized a novel low melting biodegradable hydrophilic multiblock copolymer composed of poly(ethylene glycol) and poly(ε-caprolactone) to allow extrusion at relatively low temperatures. We investigated the extrusion characteristics of this polymer and explored a strategy how to control the release of the model protein lysozyme from small diameter extruded implants. It was found that the polymer could be well extruded at temperatures as low as 55 °C. Moreover, lysozyme remained active both during extrusion as well as during release. Lysozyme release kinetics could be tailored by the co-incorporation of an oligosaccharide, inulin, which functions as a pore-forming excipient. It was concluded that this hydrophilic multiblock copolymer has promising characteristics for the preparation by melt extrusion of protein delivery implants with a release profile that is sustained over a period of more than 7 months.
1. Introduction

Biodegradable drug delivery systems for sustained release of therapeutically active proteins have attracted much attention over the last decades. Even though knowledge about those systems is growing, there are major issues challenging formulation scientists. Obviously, maintaining protein integrity and controlling the release are essential aspects in the development of depot formulations.

Numerous encapsulation techniques have been used to prepare parenteral protein depot systems, such as microspheres, pellets, implants and hydrogels. Many of those methods involve process conditions that expose the protein to severe stresses. Examples are the exposure of the protein to aqueous-organic solvent interfaces, hydrophobic surfaces, sheer forces, and surfactants [1]. For that reason, it is essential to diminish those stresses during formulation. One of the methods offering many advantages over many other encapsulation techniques is hot melt extrusion. This is a solvent-free method, which avoids the risk of exposing the protein to an aqueous-organic solvent interphase, usually encountered in emulsification-based microencapsulation processes used in preparation of microspheres. Furthermore, it is a fast, reproducible process with good mixing capabilities [2].

Poly(lactide-co-glycolide) (PLGA) is widely used for the preparation of depot formulations by hot melt extrusion. Although PLGA has several advantages, including biocompatibility and biodegradability [3,4], it also has several drawbacks that influence the release rate and the stability of the incorporated protein. For extrusion, often temperatures higher than 90 °C are required [5]. Obviously, these high temperatures can be highly detrimental to the structural integrity of the protein. Furthermore, PLGA is relatively hydrophobic compared to most proteins and examples of detrimental effects of the interaction of proteins with hydrophobic surfaces have been described [6,7]. This interaction can lead to structural changes to proteins during manufacturing, storage and in-vivo release. Furthermore, it has been shown that degradation of PLGA gives rise to accumulation of acidic products, which lowers the pH locally [8,9]. The acidic micro-environment can cause incomplete protein release due to, for example, protein aggregation [10,11]. Moreover, the acidic degradation products catalyze degradation of the polymer, which results in an irregular release profile of the incorporated protein [12].

Besides preparation challenges, another demanding task is to control the release profile of the protein. The release kinetics depend on many factors, such as molecular weight and charge of the protein, protein loading, additives, polymer composition, and the degradation rate of the polymer [13–15]. One of the more effective strategies to control the release drug substances from polymeric matrices
is the incorporation of hydrophilic molecules as “pore formers”. These will increase the release rate of the protein from the implant by creating channels in the polymer matrix [16]. For example poly(ethylene glycol) (PEG) of different molecular weight has been used for this purpose [16–19]. Similarly, encapsulation of bovine serum albumin and D-mannitol into poly-urethane foam showed enhanced cefadroxil-release rates [16]. Gunder et al. studied the application of HPMC as a water-soluble pore former to tailor the release of various drugs [20].

The aim of this paper is to study whether we can reduce the high process temperatures required during melt extrusion by using a novel hydrophilic multiblock copolymer [21]. This novel copolymer consists of 30 wt% poly(ε-caprolactone)-PEG<sub>1500</sub>-b-poly(ε-caprolactone) [PCL-PEG<sub>1500</sub>] and 70 wt% poly(ε-caprolactone) [PCL] blocks, with molecular weights 2000 and 4000, respectively. This multiblock copolymer will be further referred to as 30[PCL-PEG<sub>1500</sub>]-70[PCL]. It is envisaged that under physiological conditions the crystalline part of PCL will be phase separated from the amorphous phase consisting of amorphous PCL and PEG in the copolymer and thus forming a phase separated multi block copolymer. The phase separated nature allows controlled swelling of the polymer, enabling continuous release of the encapsulated drug by diffusion, in contrast to PLGA where release is in general degradation controlled resulting in biphasic release. PCL based drug delivery systems have recently gained much attention in delivery of contraceptives [22]. PCL is considered a biocompatible and biodegradable polymer, which is suitable for long term sustained delivery of drugs due to its low degradation rate when compared to many other biodegradable polymers like PLGA and poly lactic acid [23,24]. PCL has a melting temperature of only 55 °C and it is therefore expected that extrusion can be performed at much lower temperature than with PLGA. Furthermore, PEG is a biocompatible, hydrophilic and water swellable polymer [17]. Therefore, it is envisaged that PEG could shield the incorporated protein from the hydrophobic PCL surface. Furthermore, we investigated the in-vitro release of a model protein, lysozyme, from 30[PCL-PEG<sub>1500</sub>]-70[PCL] implants prepared by hot melt extrusion. Lysozyme, a protein with a molecular weight of 14.7 kDa, is well characterized in literature and has been extensively used as a model protein in the preparation of protein delivery systems [25–29]. In addition, it was evaluated whether the release can be tailored by application of the oligosaccharide inulin as a pore former. Inulin has been selected because it is compatible with proteins, has stabilizing effects on proteins and it is not metabolized and non-toxic [30].
2. Materials and methods

2.1. Materials

Micrococcus lysodeikticus and lysozyme (from chicken egg white $M_w=14$ kDa) were obtained from Sigma. Inulin 4 kDa was a gift from Sensus, Roosendaal, The Netherlands. Acetonitrile (HPLC gradient grade), ethyl acetate, sodium azide (99 wt%, extra pure, Acros, Geel, Belgium), sodium chloride, di-sodium hydrogen phosphate, potassium dihydrogen phosphate, and trifluoroacetic acid were purchased from Fisher, Landsmeer, The Netherlands. ε-caprolactone, 1,4-butanediol and 1,4-dioxane were obtained from Acros, Geel, Belgium. Stannous octoate was obtained from Sigma. 1,4-Butanediisocyanate was purchased from Bayer. Deuterated chloroform and lithium bromide were obtained from Fisher; dimethyl sulfoxide (DMSO), dimethylformamide (DMF) and PEG standards (FLUKA) were purchased from Sigma.

2.2. Multiblock copolymers synthesis

Low molecular weight poly(ε-caprolactone) (PCL) and poly(ε-caprolactone)-PEG$_{1500}$-b-poly(ε-caprolactone) ([PCL-PEG$_{1500}$]) prepolymers were synthesized by standard stannous octoate catalyzed ring-opening polymerization. ε-Caprolactone was dried over CaH$_2$ and distilled under reduced pressure in a nitrogen atmosphere prior to polymerization. Poly(ethylene glycol) with a molecular weight of 1500 g/mol (PEG$_{1500}$) was dried for 17 h at 90 °C under vacuum. 1,4-Butanediol and 1,4 butanediisocianate were distilled under reduced pressure. Purity of distilled ε-caprolactone, 1,4-butanediol and 1,4-butane diisocyanate was confirmed by $^1$H NMR (CDCl$_3$).

To prepare PCL with a target molecular weight of 4000 g/mol, 158.6 g (1.39 mol) of ε-caprolactone was introduced into a three-necked bottle under nitrogen atmosphere and 3.7 g (42.1 mmol) of 1,4-butanediol was added to initiate the ring-opening polymerization. Stannous octoate was used as a catalyst at a concentration of $9.72 \times 10^{-5}$ mol/mol monomer. The mixture was magnetically stirred for 71 h at 140 °C and subsequently cooled down to room temperature.

PCL-PEG$_{1500}$ prepolymer with a target molecular weight of 2000 g/mol, was synthesized in a similar way using 62.7 g (0.55 mol) of ε-caprolactone, 189.7 g (0.13 mmol) of PEG$_{1500}$ and catalyst concentration of $1.92 \times 10^{-4}$ mol/mol monomer. The mixture was magnetically stirred for 164 h at 130 °C and subsequently cooled down to room temperature.
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PCL and PCL-PEG\textsubscript{1500} prepolymers were then chain-extended with 1,4-butanediisocyanate to prepare 30[poly(ε-caprolactone)-PEG\textsubscript{1500}-poly(ε-caprolactone)]\textsubscript{2000}-b-70[poly(ε-caprolactone)]\textsubscript{4000} multiblock copolymer (30[PCL-PEG\textsubscript{1500}]-70[PCL]) (Figure 1). 18.91 g (4.74 mmol) of PCL and 8.00 g (4.00 mmol) of PCL-PEG\textsubscript{1500} were introduced into a three-necked bottle under nitrogen atmosphere. 65 ml of dry 1,4-dioxane (distilled over sodium wire) was added to a polymer concentration of 30 wt\% and the mixture was heated to 80°C to dissolve the prepolymers. 1.23 g (8.76 mmol) of 1,4-butanediisocyanate was added and the reaction mixture was stirred mechanically 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. The residual 1,4-dioxane content, as measured by GC headspace, was less than 200 ppm, showing effective removal of 1,4-dioxane by vacuum drying.

![Figure 1](image-url)

**Figure 1. Schematic drawing of the chain-extension reaction of PCL and PCL-PEG\textsubscript{1500} with 1,4-butanediisocyanate and formation of multiblock copolymer with randomly distributed PCL-PEG and PCL blocks, linked by chain-extender, and OH end groups.**

2.3. Polymer and implant characterization

\textsuperscript{1}H NMR was used to determine monomer conversion, monomer ratios, number average molecular weight and overall chemical composition of the polymer. \textsuperscript{1}H NMR was performed on a VXR Unity Plus NMR Machine (Varian, California, USA) operating at 300 MHz. The d\textsubscript{1} waiting time was set to 20 s, and the number of scans was 16 - 32. \textsuperscript{1}H NMR samples were prepared by dissolving 10 mg of polymer into 1 ml of deuterated chloroform, and the spectrum was determined from 0 - 8 ppm using CHCl\textsubscript{3} present as trace element in CDCl\textsubscript{3} as reference.

Monomer conversion, i.e. residual ε-caprolactone content, of the prepolymers was calculated from the peaks of the O-CH\textsubscript{2}CH\textsubscript{2}CH\textsubscript{2}CH\textsubscript{2}C(O)O methylene groups of PCL and monomer ε-caprolactone at δ 2.2 - 2.5 and δ 2.65, respectively. The experimental number average molecular weight (Mn) of the PCL prepolymer was determined by \textsuperscript{1}H NMR using the peaks of the methylene end groups of PCL.
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at δ 3.6 - 3.7 and the O-CH$_2$CH$_2$CH$_2$CH$_2$C(O)O methylene group of PCL at δ 2.2 - 2.5. The experimental Mn of the PCL-PEG$_{1500}$ prepolymer was determined by the combined peak of methylene end groups of poly(ε-caprolactone) and methylene groups –CH$_2$CH$_2$-O of PEG at δ 3.6 - 3.7 and the O-CH$_2$CH$_2$CH$_2$CH$_2$CH$_2$C(O)O methylene group of PCL at δ 2.2 - 2.5. The theoretical Mn of the PCL and PCL-PEG$_{1500}$ prepolymer was determined based on in-weights and monomer conversion.

$^1$H NMR was also used to verify the overall caprolactate / PEG (CL / PEG) monomer ratio of the multiblock copolymer and was calculated from and the O-CH$_2$CH$_2$CH$_2$CH$_2$CH$_2$C(O)O methylene group of PCL at δ 2.2 - 2.4 and the –CH$_2$CH$_2$-O methylene groups of PEG at δ 3.6-3.7. The intrinsic viscosity of the polymer dissolved in chloroform was determined by measuring 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/0.53 mm. Samples were prepared in DMSO. 1,4-Dioxane content was determined using 1,4-dioxane calibration standards.

Molecular weight of the multiblock copolymer was determined using size exclusion chromatography (SEC-HPLC, Waters, Breeze, USA). The sample (0.01 g) was dissolved in DMF (1 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 molecular weights were then calculated with the aid of the PEG standards calibration curve.

Thermal characteristics of PEG$_{1500}$, the prepolymer PCL, the multiblock copolymer before extrusion and the lysozyme/inulin loaded implants were measured by standard differential scanning calorimetry (DSC) using a Q2000 differential scanning calorimeter (TA Instruments, Ghent, Belgium). Samples of 5 - 10 mg were heated from –85 °C to 100 °C at a rate of 2 °C /min. During the measurement, the sample cell was purged with nitrogen gas. The heat flow was used for determination of the glass transition temperature (midpoint of the transition, determined via inflection) and melting temperature (maximum of endothermic peak). The heat of fusion was calculated from the surface area of the melting endotherm.

X-ray Power Diffraction (XRPD) was used to characterize the physical state of PEG$_{1500}$, the prepolymer PCL and PCL-PEG$_{1500}$; the multiblock copolymer 30[PCL-PEG$_{1500}$]-70[PCL], and the lysozyme/inulin 1/1 w/w loaded 30[PCL-PEG$_{1500}$]-70[PCL] implants. XRPD was performed using a D2-Phaser (Bruker
AXS, Karlsruhe, Germany) using CuKα radiation with a wavelength of 1.54 Å, at a voltage of 30 kV and current of 10 mA. All samples were prepared on a flat silica zero background sample holder covered with layer of Vaseline and scanned from 5 - 40° 2θ using a step size of 0.02° 2θ and a time per step of 1s. The scans were analyzed with the Diffraction Evaluation V 2.1 software package (Bruker AXS, Karlsruhe, Germany).

2. 4. Spray-drying

Lysozyme was incorporated in inulin by spray-drying using a B-290 mini spray drier (Büchi Labortechnik AG, Flawil, Switzerland). Lysozyme was dissolved in demineralized water (5 mg/ml) and inulin was added in different concentrations to obtain lysozyme/Inulin (Lys/Inulin) weight ratios of 1/0, 1/1, 1/2, and 1/3. Samples were spray dried under the following conditions: atomizing pressure of 55 psi, liquid feed rate of 2.5 ml/min, an inlet temperature of 100 °C and the outlet temperature was determined to be 60 ± 3 °C. Samples were stored over silica in a desiccator for at least 1 day prior to melt extrusion.

2. 5. Particle size measurements

The particle size distribution of lysozyme as received and of the spray-dried powders was determined by laser diffraction analysis. Small amounts of material (20 - 30 mg) were dispersed using a RODOS dispersing system at 3.0 bar. The particle size distribution was determined with a KA laser diffraction apparatus (Sympatec GMBH, Clausthal-Zellerfeld, Germany), equipped with a 175 mm lens. Calculations were based on Fraunhofer theory.

2. 6. Hot melt extrusion

Hot melt extrusion was performed using a HAAKE MiniLab Rheomex CTW5 (Thermo-Electron) co-rotating twin-screw extruder equipped with conveyer belt to stretch the molten material. The extrusion process was performed with a screw speed of 15 - 20 rpm, developing the torque of 8-16 Nm. Powder blends (5.5 g) containing 10 wt% lysozyme were prepared by manually mixing lysozyme as received or spray dried Lys/Inulin with 30[PCL-PEG1500]-70[PCL] at different ratios (see Table 1) after which they were fed into the preheated barrel of the extruder (set temperature of 55 °C). A cylindrical die of 0.5 mm was used resulting in implants with a diameter of 0.35 mm (± 0.05), as measured with an in-line laser. Extruded samples were stored in a freezer at -20 °C until further evaluation.
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Table 1. The Lys/Inulin ratios used in preparation of implants. The lysozyme loading was 10 wt% in each formulation; * Spray dried.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Lys/Inulin weight ratio</th>
<th>Inulin loading (wt%)</th>
<th>30[PCL-PEG&lt;sub&gt;1500&lt;/sub&gt;]-70[PCL] loading (wt%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lys as received</td>
<td>1/0</td>
<td>0</td>
<td>90</td>
</tr>
<tr>
<td>Lys/Inulin 1/0 *</td>
<td>1/0</td>
<td>0</td>
<td>90</td>
</tr>
<tr>
<td>Lys/ Inulin 1/1 *</td>
<td>1/1</td>
<td>10</td>
<td>80</td>
</tr>
<tr>
<td>Lys/Inulin 1/2 *</td>
<td>1/2</td>
<td>20</td>
<td>70</td>
</tr>
<tr>
<td>Lys/Inulin 1/3 *</td>
<td>1/3</td>
<td>30</td>
<td>60</td>
</tr>
</tbody>
</table>

2.7. Extraction of protein from extrudates

Lysozyme was extracted from the implants after extrusion to determine the content, the quality of mixing of the extruder, and to evaluate protein activity. Randomly chosen implant fractions from the beginning, the middle, and the end of the process were dissolved in 1.5 ml of ethyl acetate as described previously [31] and centrifuged for 15 min at 22,000g (Hermle Z323K, Wehingen, Germany). The dissolved polymer was removed with the supernatant. The process was repeated 3 times, after which the samples were dried in a vacuum oven (Fistreem, Loughborough, United Kingdom) at room temperature for 48 h to remove traces of ethyl acetate. After drying, the protein pellets were dissolved in 100 mM phosphate buffer, pH 7.4. The lysozyme concentration was determined by HPLC as described in section 2.8 and the activity of the protein was determined as described in a section 2.9. Control experiments using physical mixtures of polymer and protein showed that by the extraction procedure, all of the protein was recovered and that the extraction procedure did not affect the biological activity of the protein.

2.8. In-vitro release kinetics

The in-vitro release of lysozyme was evaluated in 100 mM phosphate buffer, (9.1 mM NaCl), pH 7.4 (containing 0.02 wt% of NaN₃ to prevent bacterial growth). Approximately 30 mg samples were incubated in 1.2 ml of buffer in a shaking water bath at 37 °C. At each sampling time point, 1 ml of release medium was withdrawn and replaced with 1 ml of fresh medium. The lysozyme concentration was measured with HPLC at a wavelength of 280 nm using a HPLC Ultimate 3000, equipped with
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C18 ProZap LC/MS reversed phase column (20 x 4.6 mm, 1.5 µm) and a gradient system consisting of 0.1 vol% trifluoroacetic acid in acetonitrile (A) and 0.1 vol% trifluoroacetic acid in Millipore water (B). The solvent flow rate was 1 ml/min and the gradient was applied for 6 min, using 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 lysozyme retention time was 1.19 min. Data were analyzed with Chromeleon software.

In order to study the mechanism of protein release from the polymer matrices, the results of the in-vitro release experiments were fitted in different kinetic equation’s, i.e. zero order (cumulative percentage of protein released vs time):

\[
Q_t = Q_0 + K_0 \times t
\]

where \( Q_t \) is the amount of drug released in time \( t \), \( Q_0 \) is the initial amount of drug in the solution and \( K_u \) is the zero order release constant.

First order (log percentage of protein released vs time):

\[
\log Q_t = \log Q_0 + \frac{K_1 t}{2.303}
\]

where \( Q_t \) is the amount of drug released in time \( t \), \( Q_0 \) is the initial amount of drug in the solution and \( K_1 \) is the first order release constant.

Higuchi’s model (percentage of protein released vs square root of time) [32,33]:

\[
Q_t = K_H \times t^{1/2}
\]

where \( Q_t \) is a cumulative amount of drug released at time \( t \), and \( K_H \) is the Higuchi constant.

For all models, coefficients of determinations \( R^2 \), as a measure of similarity between the data set and the equations, were calculated by regression analysis.

2.9. Biological activity of lysozyme

The biological activity of lysozyme was measured with a turbidimetric assay [34] adapted for a plate-reader. Test samples of 20 µl (of which the lysozyme concentration was measured by HPLC) and calibration samples (0 - 125 µg/ml) were pipetted into the wells of a 96 wells plate. The bacteria M. lysodeikticus were suspended in 66 mM phosphate buffer pH 6.2, to obtain a concentration of 2.5 mg/ml. 200 µl of bacterial suspension was added to each well. Lysozyme is capable of disrupting bacterial walls, leading to a decrease in the turbidity of the suspension. The decrease
of turbidity was determined by measuring the absorbance at an arbitrarily chosen wavelength of 415 nm during 4 min at 37 °C using a plate reader (Benchmark Plate reader, Bio-Rad, Hercules, USA). The rate of the decrease of absorption of a test sample was compared to a calibration curve and was used to calculate the activity of the protein.

2. 10. Scanning electron microscopy (SEM)

The morphology of the implants was investigated with scanning electron microscopy (SEM, JEOL, JSM 6301-F Microscope, JEOL, Japan). Implants were attached to a double-sided carbon tape and coated with gold - palladium. All samples were examined before release and after 1 day of release, to study possible pore formation.

3. Results

3. 1. Polymer synthesis and characterization

$^1$H NMR analysis of the PCL prepolymer showed a monomer conversion of 99.8 % and a molecular weight (Mn) of 4095 g/mol, which was close to the theoretical value of 3990 g/mol as calculated from in-weights and monomer conversion. For the prepolymer PCL-PEG$_{1500}$ a monomer conversion of 98.8 % and a Mn of 1970 g/mol were calculated from the $^1$H NMR spectrum, which was close to the theoretical Mn value as calculated from in-weights and monomer conversion (1960 g/mol).

$^1$H NMR was used to verify the overall caprolactate/polyethylene glycol (CL/PEG) monomer ratio of the multiblock copolymer. The overall CL/PEG molar ratio of the 30[PCL-PEG$_{1500}$]-70[PCL] multiblock copolymers, as determined by $^1$H NMR was found to be 41.6 mol / mol, which was close to the theoretical CL/PEG molar ratio of 44.0 as calculated from in weights. The 30[PCL-PEG$_{1500}$]-70[PCL] multiblock copolymer had an intrinsic viscosity of 0.66 dl/g. SEC showed an Mn of 17.6 kg/mol, relative to PEG standards, and polydispersity index (Mw/Mn) of 1.70.

3. 2. XRPD

The clear peaks in the diffractograms of PEG$_{1500}$ and the prepolymer showed that PEG$_{1500}$, PCL and PEG$_{1500}$ in PCL-PEG$_{1500}$ prepolymer, were crystalline before synthesis of multiblock copolymer (Figure 2). The diffractogram of the
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30[PCL-PEG\textsubscript{1500}]-70[PCL] multiblock copolymer was almost identical to that of the PCL prepolymer, while the peaks from PEG\textsubscript{1500} of the prepolymer were absent in the multiblock copolymer, indicating that the crystalline structure of PEG\textsubscript{1500} was lost during chain-extension of the prepolymers into multiblock copolymer. The diffractogram of the Lys/Inulin 1/1 loaded implants resembled to the 30[PCL-PEG\textsubscript{1500}]-70[PCL] diffractogram before extrusion, indicating that the addition of 20 wt% of solid sample and the hot melt extrusion did not affect recrystallization of the multiblock copolymer. Additionally, no extra peaks from Lys/Inulin were noticed, showing that as expected the protein/sugar phase was completely amorphous [35].

![Figure 2. X-ray powder diffraction patterns of PEG\textsubscript{1500}, PCL-PEG\textsubscript{1500} and PCL prepolymers, 30[PCL-PEG\textsubscript{1500}]-70[PCL] multiblock copolymer and Lys/Inulin 1/1 loaded implants.](image)

3. 3. DSC

To confirm the results of the XRPD measurements and to obtain more quantitative data about crystallinity of Lys/Inulin loaded polymer implants, the samples were also analyzed by DSC. Thermograms are shown in Figure 3. PCL-PEG\textsubscript{1500} prepolymer
showed a melting endotherm with three overlapping peaks, main peak at 43 °C and two minor peaks at 30 °C and 39 °C. These peaks are attributed to melting of different crystal modifications of PEG [36]. The heat of fusion (ΔH) of all three peaks together was 102 J/g, indicating 82 % crystallinity of PEG$_{1500}$ in the prepolymer (assuming that the ΔH of fully crystalline PEG is 165.5 J/g [37]). The melting temperature (Tm) of the PCL prepolymer was 57 °C with a ΔH of 94 J/g, representing a degree of crystallinity of approximately 67 %, assuming a ΔH of 139.3 J/g for fully crystalline PCL [38]. The DSC thermograms of the 30[PCL-PEG$_{1500}$]-70[PCL] showed two Tm values at around 40 °C and 52 °C and a small glass transition (Tg) at around -60 °C. The large melting peak of the multiblock copolymer at around 52 °C is attributed to the melting of PCL and the melting peak at around 40 °C most likely represents melting of PEG. The melting peaks of PCL and PEG partially overlapped and therefore only an estimation of the degree of crystallinity can be given. The degree of crystallinity of the PCL segment in the multiblock copolymer was estimated to be around 70 %. The ΔH of PEG was only around 1 J/g, showing that the PEG$_{1500}$ segment in the PCL-PEG$_{1500}$ prepolymer lost most of its crystallinity after conversion into multiblock copolymer, as it was also indicated by XRPD. Thermograms for lysozyme (/inulin) loaded 30[PCL-PEG$_{1500}$]-70[PCL] implants were very similar, showing a Tg around -62 °C and a Tm around 53 °C (Table 2). The ΔH of PEG in implants was difficult to quantify due to a very small surface area of the transition. For all five implants, the crystallinity of the PCL segments of the multiblock copolymer was found to be 40 – 50 % (Table 2), which was lower than the crystallinity of the 30[PCL-PEG$_{1500}$]-70[PCL] before extrusion.

Figure 3. Thermograms of prepolymers PCL, PCL-PEG$_{1500}$, multiblock copolymer 30[PCL-PEG$_{1500}$]-70[PCL] and Lys/Inulin 1/1 loaded implant.
Table 2. Tg; Tm and ΔH of 30[PCL-PEG_{1500}]-70[PCL] multiblock copolymer and implants with particle size distribution of spray dried samples and lysozyme as received. Span is calculated using following equation: Span = (X_{90} - X_{10}) / X_{50}, where X_{10}, X_{50} and X_{90} indicate the volume percentage of particles (10 %, 50 % and 90 % undersize respectively).

<table>
<thead>
<tr>
<th>(protein + sugar)/polymer weight ratio</th>
<th>Thermal properties</th>
<th>Particle size measurements (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tg (°C)</td>
<td>Tm (°C)</td>
</tr>
<tr>
<td>30[PCL-PEG_{1500}]-70[PCL] before extrusion</td>
<td>0/100</td>
<td>-60.9</td>
</tr>
<tr>
<td>Lys as received</td>
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</tr>
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<td>Lys/Inulin 1/3</td>
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</table>
3.4. Particle size measurements

Particle size distribution data of spray-dried samples and lysozyme as received are shown in Table 2. For all spray-dried formulations a narrow and uniform size distribution was observed, while lysozyme as received had a broader size distribution. In addition, the particle size of the spray-dried samples was much smaller than lysozyme as received.

3.5. Hot melt extrusion

As expected, 30[PCL-PEG\textsubscript{1500}]-70[PCL] could be extruded at a temperature of 55 °C, which was slightly higher than the melting temperature of the multiblock copolymer. Extraction of the protein from randomly taken samples showed uniform protein loading, indicating excellent mixing. It was shown that the average protein content in the product (10.05 ± 0.01 wt%, n=3) did not substantially deviate from the theoretical loading (10 wt%). The very small standard deviation shows that the distribution of protein in the implant is homogeneous.

3.6. In-vitro lysozyme release

The in-vitro release of lysozyme was studied over a period of 260 days (Figure 4). As can be seen, the biphasic release, which is often encountered with PLGA-based release systems, was not observed. Instead, after an initial burst, a gradual release of the protein was observed. The cumulative amount of protein released at 4 h was considered as a burst release. The release rate of lysozyme after 4 h largely increased upon increasing the inulin content. The release of lysozyme from the Lys/Inulin 1/2 and Lys/Inulin 1/3 formulation was very fast as all protein was released within 12 and 3 days, respectively. The formulation of Lys/Inulin 1/1 showed a much slower release, 84 ± 4 % of protein was released within 260 days. Implants consisting of spray dried Lys/Inulin 1/0 released only 39 ± 3 % of protein during duration of the study. Interestingly, formulation of 10 wt% Lys as received showed a faster release rate than spray-dried lysozyme. After a burst release of 19 ± 1 %, around 75 ± 2 % was released within 260 days. To obtain a better understanding about the drug release mechanism, the release data were fitted into various kinetic models (Table 3). The release data of the Lys as received and Lys/Inulin 1/0 formulations showed the best fit in the Higuchi model, which is indicative for diffusion-controlled release. On the other hand, the release data of the Lys/Inulin 1/1 was quite poor for all three models, however, the best fit was for the Higuchi model as well. Also the release data of the Lys/Inulin 1/2 and Lys/Inulin 1/3 formulation showed a poor fit in all three
models. However, in these cases, the best fit was the first order model ($R^2 = 0.895$, $R^2 = 0.677$), which is indicative for dissolution-controlled release.

![Figure 4. The in-vitro release of lysozyme in 100 mM phosphate buffer, pH 7.4 at 37 °C for 260 days, (n=3). The initial 24 h release (upper figure) is shown in hours.](image)

Table 3. Coefficient of determination ($R^2$) obtained from release data of various kinetic models.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Zero order</th>
<th>First order</th>
<th>Higuchi</th>
</tr>
</thead>
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<tr>
<td>Lys as received</td>
<td>0.812</td>
<td>0.931</td>
<td>0.957</td>
</tr>
<tr>
<td>Lys/Inulin 1/0</td>
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<td>0.963</td>
<td>0.997</td>
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<td>0.895</td>
<td>0.742</td>
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<tr>
<td>Lys/Inulin 1/3</td>
<td>0.197</td>
<td>0.677</td>
<td>0.352</td>
</tr>
</tbody>
</table>

3.7. Protein activity

Even though lysozyme is often considered as a very stable protein [39], many studies suggest that incomplete lysozyme release from polymeric depot formulations is due to denaturation of the protein or absorption of the protein to the polymer surface [27,40]. Therefore, the activity of lysozyme was studied immediately after
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spray-drying, the hot melt extrusion process and during release. These experiments showed that lysozyme remained fully active after spray-drying and extrusion. In addition, during incubation of the implants in phosphate buffer, lysozyme that was released from the implant fully preserved its activity until 170 days, independent of the formulation. After 170 days and until the end of the study (260 days) the concentration of released lysozyme could still be measured accurately by HPLC but was in some cases too low to be precisely analyzed by enzymatic activity assay due to non-linearity of the assay at these low concentrations. Furthermore, the activity of lysozyme in some samples seemed to be decreased to 80 %.

3. 8. SEM

SEM images were taken after extrusion and after 1 day of incubation of the implants in phosphate buffer. As shown in Figure 5a the formulation with 10 wt% Lys as received showed numerous, large particles on the surface of implants before incubation. After 1 day of release, these particles had disappeared and large pores were formed at the surface of the implant indicating that these particles consisted of lysozyme (Figure 5b). Also the implant containing the Lys/Inulin 1/0 (spray dried lysozyme) formulation showed particles on its surface before incubation. However, these particles were much smaller (Figure 5c). Also in this case these particles had disappeared after 1 day of incubation while small pores were formed at the surface (Figure 5d). The pore size distribution was in agreement with the particle size distribution of spray-dried lysozyme (Table 2), indicating that the pores were formed by the dissolving lysozyme. As expected, the number of pores increased with increased loading as shown on SEM images of Lys/Inulin 1/3 formulation after 1 day of incubation (Figure 5e).
4. Discussion

In this study, we intended to evaluate the hydrophilic, phase separated 30[PCL-PEG_{1500}]70[PCL] multiblock copolymer for its suitability to prepare protein loaded implants by low temperature extrusion. The phase separated nature of the multiblock copolymer, i.e. the presence of both (water-insoluble) crystalline PCL domains and PEG containing amorphous domains, ensures good mechanical integrity in-vivo,
controlled swelling and thus continuous release of encapsulated compounds. The presence of low melting PCL crystals allows for the preparation of implants by low temperature extrusion, which minimizes risk of denaturation of encapsulated proteins.

For the multiblock copolymer synthesis, firstly, the prepolymer PCL and PCL-PEG\textsubscript{1500} were synthesized by ring opening polymerization of $\varepsilon$-caprolactone initiated by 1,4-butanediol and polyethylene glycol (PEG $M_w$=1500), respectively, catalyzed by stannous octoate. For both prepolymer, the Mn as determined by $^{1}$H NMR agreed well with the theoretical values, showing that prepolymer with well-defined composition were obtained.

$30[\text{PCL-PEG}_{1500}]-70[\text{PCL}]$ multiblock copolymer was prepared by chain-extension of PCL-PEG\textsubscript{1500} and PCL with 1,4-butanediisocyanate in 1,4-dioxane at 80 °C. The CL/PEG molar ratio from $^{1}$H NMR agreed well (within the error of the $^{1}$H NMR method which is about 5 - 10%) with molar ratio based on in-weights (44.0 vs. 41.6 mol/mol).

XRPD data showed that PEG\textsubscript{1500} of the PCL-PEG\textsubscript{1500} prepolymer lost most of its crystalline structure during synthesis of $30[\text{PCL-PEG}_{1500}]-70[\text{PCL}]$ but that PCL originating from the PCL prepolymer was still (partially) crystalline in the multiblock copolymer. Since XRPD provides only qualitative information, the samples were also analyzed by DSC to quantify the crystallinity of PCL. The phase-separated nature of the $30[\text{PCL-PEG}_{1500}]-70[\text{PCL}]$ multiblock copolymer was shown by the presence of a $T_m$ for crystalline PCL at 57 °C and a $T_g$ at -60 °C representing an amorphous phase. The amorphous phase of the $30[\text{PCL-PEG}_{1500}]-70[\text{PCL}]$ multiblock copolymer most probably consisted of phase-mixed amorphous PEG and PCL [41,42]. Further, a small melting endotherm at 42 °C was present, which is attributed to melting of crystalline PEG. The very low melting enthalpy for PEG1500 showed that the PEG crystals were almost completely transformed into the amorphous state in the multiblock copolymer. This very small crystalline fraction of PEG detected by DSC was probably below the detection limit of XRPD. Note that any PEG crystallinity will be lost upon immersion of $30[\text{PCL-PEG}_{1500}]-70[\text{PCL}]$ under physiological conditions, i.e. in an aqueous environment at 37 °C. The $T_m$ values of both PEG and PCL in $30[\text{PCL-PEG}_{1500}]-70[\text{PCL}]$ were lower than in the prepolymer, which is most likely due to formation of less dense crystals, caused by partial phase-mixing of the PCL and PCL-PEG\textsubscript{1500} blocks and possibly also by hampered crystallization when the chain ends of the prepolymer are embedded in the multiblock copolymer. Lys/(Inulin) loaded implants showed similar $T_m$ and $T_g$ as the non-extruded $30[\text{PCL-PEG}_{1500}]-70[\text{PCL}]$ multiblock copolymer, showing that the phase separated morphology was maintained after extrusion.
Hot melt extrusion experiments showed that due to the melting temperature of poly(\(\varepsilon\)-caprolactone) blocks at 50 - 53 °C, the multiblock copolymer could indeed be extruded at a relatively low temperature of 55 °C. The crystallinity of PCL decreased after extrusion (70 % vs. 40 – 50 %), but the remaining degree of crystallinity was still sufficient to obtain non-sticky Lys (/Inulin) implants with good handling properties.

The suitability of the multiblock copolymer to be used as matrix material for the controlled release of proteins was investigated. In addition, in order to study the ability of co-incorporated inulin to act as release modifier, various amounts of inulin (co-processed with the protein first by spray drying) were co-incorporated into the polymer matrix while keeping the amount of protein the same. It is known that degradation of PCL and its copolymers takes longer than 2 years under both in-vitro and in-vivo conditions [43,44], therefore we do not expect substantial degradation of this multiblock copolymer within the time frame of this study. For that reason, we presume that release is primarily driven by other mechanisms than degradation. It was observed that the initial release of lysozyme from the polymer implants was highly dependent on the particle size of the protein and on the total solid loading and thus on the protein/inulin ratio of the spray dried samples. The formulation consisting of 10 wt% spray dried lysozyme (Lys/Inulin 1/0) exhibited the smallest burst release. Since the surface morphology after one day of incubation, as observed on SEM images, showed small pores with the size correlating to the particle size of the spray dried powder (Table 2), the burst release can be ascribed to dissolution of lysozyme particles at the surface of the implant. The formulation containing lysozyme as received displayed a much larger burst release. Similar to the Lys/Inulin 1/0 formulation, a burst release is expected to be the result of dissolution of the particles at the surface. Since the size of these particles is much larger than the spray-dried particles, the burst release is higher. After the burst release, the release rate of lysozyme from the implant containing lysozyme as received was somewhat higher than from the Lys/Inulin 1/0 formulation, most likely due to the larger surface area of the implant containing lysozyme as received due to the dissolution of large lysozyme particles during the burst release.

After 70 days however, the release of lysozyme from the implant containing either spray-dried lysozyme (Lys/Inulin 1/0) or lysozyme as received was similar indicating that in this later stage the release was not dependent on the particle size. At a loading of 10 wt% it can be assumed that only few lysozyme particles are connected forming channels in the implant through which the protein can diffuse out. Therefore, the release will be predominately governed by diffusion of the protein through the polymer matrix. Since the diffusion rate of the protein through the polymer matrix will be independent of the original particle size, the release rate will be similar. When the total loading is increased more lysozyme/inulin particles will be connected forming
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channels in the implant through which the protein can diffuse out. Therefore, the release will be governed by both the diffusion of lysozyme through these channels and diffusion through the polymer. Because the diffusion of the protein through the channels will be much faster than through the polymer matrix, the release rate will be increased. This phenomenon is clearly visible as the release from the Lys/Inulin 1/1 is much faster than from the Lys/Inulin 1/0 implant formulations. That the release of the protein from these three formulations is controlled by diffusion is supported by the best fit of their release data in the Higuchi model. When the total loading exceeds a certain threshold, all lysozyme/inulin particles will form an interconnected (percolating) structure, which will give rise to a very fast release as the release is now governed by protein dissolution and diffusion through the channels. This was clearly found for the Lys/Inulin 1/2 and Lys/Inulin 1/3 formulations. The release data of these two formulations showed a poor fit in all three models applied in this study with the best fit for the first order model. This confirms that the release could indeed be governed by a combination of dissolution and diffusion. In summary, these findings show that the particle size of the protein and the loading of the polymer are factors that need to be taken into account when designing controlled release formulations and that inulin can be successfully used as a release enhancer. This approach can be highly beneficial in the design of controlled release systems where only a small amount of a highly potent protein is to be incorporated.

Lysozyme remained fully active after extrusion, which was shown by extraction of protein with ethyl acetate. Even though it is known that organic solvents could create detrimental conditions by changing the proteins’ secondary structure, it has previously been shown that ethyl acetate is less harmful than other organic solvents [45,46]. Therefore, we considered it safe to use ethyl acetate in these experiments. In fact, the finding that extraction yielded a protein, which was fully active confirms that ethyl acetate can indeed be safely used for the extraction procedures. Furthermore, during 170 days lysozyme remained fully active independent on the formulation. The observed remaining activity of lysozyme is not surprising when taking into account that lysozyme is a relatively stable model protein [28,47]. However, when the controlled release of a less stable protein is envisaged, the pre-incorporation of the protein in a matrix of inulin glass would be beneficial, as it stabilizes the protein [48]. Additionally, the possibility to extrude proteins at temperatures as low as 55 °C will be advantageous compared to significantly higher temperatures (80 - 100 °C) that are required to extrude PLGA based implant formulations. Indeed, in preliminary experiments we showed that highly heat-labile protein alkaline phosphatase remained fully active after hot melt extrusion with 30[PCL-PEG\textsubscript{1500}]-70[PCL] at 55 °C while the protein lost half of its enzymatic activity after hot melt extrusion with PLGA at 90 °C (data not shown).
5. Conclusions

In this study, we synthesized a novel hydrophilic multiblock copolymer composed of amorphous PCL-PEG$_{1500}$ and semi-crystalline PCL blocks and evaluated this polymer as a matrix material for the controlled release of proteins. We demonstrated that the polymer can be extruded into implants at a temperature as low as 55 °C. Moreover, phase separation of the polymer was still observed after incorporation of protein or protein/sugar material after hot melt extrusion. *In-vitro* studies showed that no biphasic release profiles were observed, which are often encountered with PLGA-based release systems, but continuous releases is obtained close to first order kinetics after an initial burst. Furthermore, we showed that by co-incorporation of inulin as a pore-forming excipient, the release of the model protein lysozyme from polymer matrixes can be tailored. Lysozyme remained 80 % active during at least 6 months of release. As shown here, not only the total load but also the particle size of the protein should be considered when designing controlled release device. Taken together, the novel multiblock copolymer can be applied as a matrix for low temperature extrusion of implants (or other dosage forms) for the controlled release of proteins.

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References

Chapter 4


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