Protein release from water-swellable poly(d,l-lactide-PEG)-b-poly(-caprolactone) implants

Stanković, Milica; Hiemstra, Christine; de Waard, Hans; Zuidema, Johan; Steendam, Rob; Frijlink, Henderik W.; Hinrichs, Wouter L. J.

Published in:
International Journal of Pharmaceutics

DOI:
10.1016/j.ijpharm.2015.01.007

Publication date:
2015

International Journal of Pharmaceutics 480 (2015) 73–83

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Protein release from water-swellable poly(D,L-lactide-PEG)-*b*-poly(ε-caprolactone) implants

Milica Stanković [a,*], Christine Hiemstra [b], Hans de Waard [a], Johan Zuidema [b], Rob Steendam [b], Henderik W. Frijlink [a], Wouter L.J. Hinrichs [a]

[a] Department of Pharmaceutical Technology and Biopharmacy, University of Groningen, A. Deusinglaan 1, 9713 AV, Groningen, The Netherlands
[b] InnoCore Pharmaceuticals, L.J. Zielstraweg 1, 9713 GX, Groningen, The Netherlands

ARTICLE INFO

Article history:
Received 30 July 2014
Received in revised form 26 December 2014
Accepted 4 January 2015
Available online 6 January 2015

Keywords:
Polymer implants
Hot melt extrusion
Lysozyme
Bovine serum albumin
Protein–polymer interaction
Polymer degradation

ABSTRACT

In this study, water-swellable multiblock copolymers composed of semi-crystalline poly(ε-caprolactone) [PCL] blocks and amorphous blocks consisting of poly(D,L-lactide) (PDLLA) and poly(ethylene glycol) (PEG) [PDLLA–PEG] were synthesized. The block ratio of these [PDLLA–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 (Stanković et al., 2014). It was shown that the initial degradation rate of the [PDLLA–PEG]-*b*-[PCL] multiblock copolymers increased with increasing the content of amorphous [PDLLA–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 [PDLLA–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 [PDLLA–PEG]-*b*-[PCL] copolymers was incomplete, which is not acceptable for any application of these polymers. Besides, [PCL–PEG]-*b*-[PCL] copolymers 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 [PDLLA–PEG] blocks from the degrading polymer.

1. Introduction

In recent years, there has been a growing interest in the application of polymers as matrices for prolonged-release drug delivery systems (DDS).

From the mid 1970's, more and more research has been done on biodegradable polymers as an alternative to non-biodegradable polymers (Freiberg and Zhu, 2004). Contrary to non-biodegradable polymers that often require surgical removal, biodegradable polymers are susceptible to degradation in the body into small molecules either by chemical or enzymatic hydrolysis or both (Park et al., 2005). Among this class of polymers, biodegradable 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 (Vert, 2005). Moreover, the biodegradable polyesters should provide a suitable microenvironment for the encapsulated drug and avoid any significant changes in pH due to accumulation of acidic degradation products, they should exhibit control over the release rate and they should possess sufficient loading capacity for the incorporated molecules and their therapeutic applications. 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 (Jain, 2000; Lucke et al., 2000; Seyednejad et al., 2011; Vilar et al., 2012).

Prolonged release polymeric DDS may appear as microspheres, gels, transdermal patches, intra-vaginal rings or implants. The solid implants, discussed further in this study and intended for long-term parenteral delivery of therapeutic proteins can be prepared by hot melt extrusion (HME), with the final goal to treat various diseases. To date, to our knowledge, there is one protein-based therapeutic in

* Corresponding author. Tel.: +31 50 3633172; fax: +31 50 3632500.
E-mail address: m.stankovic@rug.nl (M. Stanković).

http://dx.doi.org/10.1016/j.ijpharm.2015.01.007
0378-5173/

the market (Lucentis®), administered with a needle once a month to treat age-related macula degeneration (Bakri et al. 2007). Therefore, there is a strong interest to develop ophthalmic drug delivery formulations with longer duration of release (6–12 months), in order to circumvent frequent ocular injections.

Despite the increased use of biodegradable polymers for various applications, there are still some problems that hinder the widespread application in DDS. 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 (Frank et al., 2005; Huang et al., 2006; Vert et al., 1991).

Poly(ε-caprolactone) (PCL) is a biocompatible polymer, which occurs either as amorphous or semi-crystalline material, depending on its molecular weight. However, PCL degrades slowly, both *in-vitro* and *in-vivo*, which in combination with its highly hydrophobic nature limits its application in DDS (Pitt et al., 1981a; Sun et al., 2006). 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(D,L-lactide) or poly(ε-caprolactone) (PCL) in combination with hydrophilic PEG (Chen et al., 2003; Li et al., 1997; Rashkov et al., 1996) offer the possibility of varying the hydrophilicity and swelling degree of the polymer and allow modulation of the release of hydrophilic drugs and degradation kinetics of the copolymer. More hydrophilic polymers generally provide better compatibility with hydrophilic proteins (Chen et al., 2008; Roach et al., 2005). 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 (Stanković et al., 2014). In a previous study, we introduced phase separated multiblock copolymers composed of crystalline regions of PCL and amorphous regions composed of PEG and PCL [PCL–PEG]-*b*-[PCL] (Stanković et al., 2014). The PCL in the polymer is thus semi-crystalline as it appears in both the amorphous and crystalline regions. The existence of one amorphous phase was supported by the presence of only one glass transition temperature. The amorphous domains of these polymers swell and dissolve after contact with water, while the crystalline PCL serves as a crosslink and prevents the complete polymer dissolution.

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 from the polymer implants 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 [PCL–PEG] blocks by water-swellable blocks composed of poly(D,L-lactide) and PEG ([PDLLA–PEG]) as to obtain faster degrading copolymer systems. The effect of the [PDLLA–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, D,L-lactide, PEG (M_w 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_{1000}-*b*-poly(D,L-lactide) [PDLLA–PEG_{1000}] prepolymers ($n = 2$) were synthesized by standard stannous octoate catalyzed ring-opening polymerization, as described previously (Stanković et al., 2013). Briefly, 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 8.40×10^{-5}/1. The mixture was magnetically stirred for 70 h at 140 °C and subsequently cooled to room temperature.

[PDLLA–PEG_{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_{1000} and molar catalyst/monomer ratio of 2.72×10^{-4}/l. The mixture was magnetically stirred for 10 days at 140 °C and subsequently cooled to room temperature.

[PCL] and [PDLLA–PEG_{1000}] prepolymers were then chain-extended with 1,4-butanediisocyanate to prepare *x*[PDLLA–PEG_{1000}]–*y*[PCL] multiblock copolymer ($n = 1$) where *x*/*y* is the [PDLLA–PEG_{1000}]/[PCL] weight ratio, being 10/90, 20/80, 30/70 or 50/50 (Table 1). [PCL] and [PDLLA–PEG_{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_{1500}]–70[PCL] have been described elsewhere (Stanković et al., 2013).

2.3. Polymer characterization

^{1}H NMR ($n = 1$) was performed on a VXR Unity Plus NMR Machine (Varian, California, USA) operating at 300 MHz and was used to determine monomer conversion, number average molecular weight (M_n) and overall chemical composition of the polymer after synthesis and during degradation, as explained previously (Stanković et al., 2013; Stanković et al., 2014).

Table 1
In weights of pre-polymers used in synthesis of x[PDLLA-PEG_{1000}]- y[PCL] multiblock copolymers.

	PDLLA–PEG_{1000} prepolymer wt%	PCL prepolymer wt%	PDLA–PEG_{1000} prepolymer In weights	PCL prepolymer In weights	BDI In weights
10[PDLLA–PEG_{1000}]–90[PCL]	10	90	10.28 g (5.09 mmol)	88.01 g (21.68 mmol)	3.22 g (22.95 mmol)
20[PDLLA–PEG_{1000}]–80[PCL]	20	80	19.84 g (9.82 mmol)	77.96 g (19.59 mmol)	3.51 g (25.05 mmol)
30[PDLLA–PEG_{1000}]–70[PCL]	30	70	28.30 g (14.15 mmol)	63.57 g (15.97 mmol)	3.79 g (27.07 mmol)
50[PDLLA–PEG_{1000}]–50[PCL]	50	50	48.42 g (23.97 mmol)	47.16 g (11.85 mmol)	5.08 g (36.24 mmol)

Monomer conversion was calculated from peaks originating from the polymer and the monomer. For [PCL], monomer conversion was calculated from the peaks of the —O—$CH_2CH_2CH_2CH_2\mathbf{CH_2}C(O)$— methine groups of [PCL] and monomer ε-caprolactone at δ 2.2–2.5 and δ 2.65, respectively. For [PDLLA–PEG_{1000}], monomer conversion was calculated from the peaks of the —O—C**H**$(CH_3)C(O)$— methine groups of PDL and monomer D,L-lactide at δ 5.1–5.4 and δ 5.0–5.1, respectively. The experimental number average molecular weight of the [PCL] prepolymer was determined by ^{1}H NMR using the peaks of the methine end groups of PCL at δ 3.6–3.7 and the —O—$CH_2CH_2CH_2CH_2\mathbf{CH_2}C(O)$— methine group of PCL at δ 2.2–2.5. The experimental M_n of the [PDLLA–PEG_{1000}] prepolymer was determined by the peak of the PDLLA methine groups —O—C**H**$(CH_3)C(O)$— at δ 5.1–5.4 and the peaks of the PEG methine —$\mathbf{CH_2CH_2}$—O at δ 3.6–3.7.

^{1}H NMR was further used to verify the overall ε-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 O—$CH_2CH_2CH_2CH_2\mathbf{CH_2}C(O)$— methine group of PCL and ε-caprolactone at δ 2.2–2.5 and δ 2.65, respectively, and the —$\mathbf{CH_2CH_2}$—O methine groups of PEG at δ 3.6–3.7. PDLLA/PEG molar ratio was calculated from the —O—C**H**$(CH_3)C(O)$— methine groups of PDLLA and D,L-lactide monomer at δ 5.1–5.4 and δ 5.0–5.1, respectively, and the —$\mathbf{CH_2CH_2}$—O methine groups of PEG at δ 3.6–3.7.

The residual 1,4-dioxane content of the multiblock copolymer ($n = 2$) was determined using a GC-FID headspace method (GC–FID Combi Sampler supplied with an Agilent Column, DB-624/30 m/0.53 mm), as described in Stanković et al. (2014).

The apparent molecular weight of the multiblock copolymers ($n = 2$) was determined using size exclusion chromatography (SEC–HPLC, Waters, Breeze, USA), as described before (Stanković et al., 2014). Polymers were detected by refractive index. The apparent M_n and apparent weight average molecular weight (M_w) were calculated with the aid of the PEG standards calibration curve.

Modulated differential scanning calorimetry (DSC) using Q2000 differential scanning calorimeter (TA instruments, Ghent, Belgium) was used to determine the thermal behavior of the multiblock copolymers. About 5–10 mg of dry material ($n = 2$) 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 (T_g, midpoint), while the total heat flow was used for determination of the melting temperature (maximum of endothermic peak, T_m) 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 ($n = 1$) were prepared from x[PDLLA–PEG_{1000}]–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_{1500}]–70[PCL] loaded with 10% Lys as listed in Table 2.

Since protein particle size may influence the release kinetics from polymer matrices (Ghalanbor et al., 2010; Stanković et al., 2013), 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). The milled BSA powder was studied using HPLC, to assess whether the additional peaks were present as a consequence of protein degradation or aggregation. Milled BSA or Lys powder were then physically mixed with polymer powder using mortar and pestle and fed into the preheated barrel of the extruder.

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

Formulation name and polymer grade	Protein	PEG content in multiblock copolymer (wt%)
Lys/10[PDLLA–PEG_{1000}]–90[PCL]	Lys	5
Lys/20[PDLLA–PEG_{1000}]–80[PCL]	Lys	10
Lys/30[PDLLA–PEG_{1000}]–70[PCL]	Lys	15
Lys/50[PDLLA–PEG_{1000}]–50[PCL]	Lys	25
BSA/10[PDLA–PEG_{1000}]–90[PCL]	BSA	5
BSA/20[PDLA–PEG_{1000}]–80[PCL]	BSA	10
BSA/30[PDLLA–PEG_{1000}]–70[PCL]	BSA	15
BSA/50[PDLLA–PEG_{1000}]–50[PCL]	BSA	25
Lys/30 [PCL–PEG_{1500}]–70[PCL]	Lys	22.5
10[PDLLA–PEG_{1000}]–90[PCL]	–	5
20[PDLLA–PEG_{1000}]–80[PCL]	–	10
30[PDLLA–PEG_{1000}]–70[PCL]	–	15
50[PDLLA–PEG_{1000}]–50[PCL]	–	25
30[PCL–PEG_{1500}]–70[PCL]	–	22.5

Extrusion was performed at 50–55 °C using a screw speed of 10–20 rpm and a torque of 4–7 N/m. 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 (Keyence lased micrometer LS-3100, with scanning head LS-3060T, Osaka, Japan). Polymer only implants, used for the degradation study were extruded similarly, without further additives. Polymer strands were cut into pieces of 2 cm × 0.35 mm (length × diameter, for the degradation study) and 1 cm × 0.35 mm (for the *in-vitro* release study) and stored at −20 °C prior to use.

2.5. In-vitro *polymer degradation*

Considering that the purpose of this study was the development of implants capable of releasing proteins during a 6 months period, the degradation of polymer only implants was evaluated during 180 days. In total, about 130 ± 5 mg 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% NaN_3) was added to each vial. The vials were then incubated in an oven at 37 °C. According to the ISO standard 15814 (The International Organization for Standardization, 1999), at various intervals, three samples were taken at each measuring point and pH was regularly measured and adjusted to pH 7.4 ± 0.2 using 1 M NaOH to avoid the potential auto-catalytic effect of polymer degradation products on further polymer degradation. 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 ($m_{wet,t}$) 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 until constant mass and weighted again ($m_{dry,t}$). Water content and mass loss were calculated using Eqs. (1) and (2). Samples were not returned to the buffer, however, they were further analyzed using ^{1}H NMR, SEC and DSC, as described in Section 2.3.

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

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

$m_{dry,0}$ is the mass of the dry sample at day 0, $m_{dry,t}$ is the mass of the dry sample at time t and $m_{wet,t}$ is the mass of the wet sample at time t.

2.6. In-vitro *protein release and quantification*

The *in-vitro* protein release was evaluated in 100 mM PBS (pH 7.4 ± 0.2, 9.1 mM NaCl, 0.02 wt% NaN_3). Approximately 30 mg of polymer implants 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 according to a modified HPLC method (Liao et al., 2001) and refreshed (n = 3). The cumulative amount of released protein within 4 h was considered as burst release. The percentage of protein release was calculated in relation to the total protein loading, which was determined by protein extraction from protein loaded implants (Section 2.7).

Lys concentrations were measured with Dionex Ultimate 3000HPLC (Thermo Scientific, Sunnyvale, CA, USA), equipped with C18 ProZap LC/MS reversed phase column (Grace Davidson, Deerfield, IL, USA) (20 × 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: 0–1 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 BEH300C4 reversed phase column (50 × 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; 4–5 min 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 (Stanković et al., 2013). 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 by HPLC 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. The total protein loading was calculated from the weighed implant amount and protein concentrations determined by HPLC.

After 30 and 105 days of the *in-vitro* protein release, polymer implants were taken from the medium, dried in a desiccator for 24 h until constant mass and extraction was performed as described above, using separately, next to ethyl acetate the following solvents: acetone, acetonitrile, DMSO/0.05 N NaOH + 0.5% SDS, dichloromethane. The protein content was measured using the protein quantification assay (Pierce 600 nm), which is compatible with the abovementioned reagents.

As control, polymer only implants without encapsulated protein taken during the degradation study (after 28 days and 128 days) were dried in a desiccator for 24 h until constant mass and the extraction was performed using both ethyl acetate and dichloromethane.

The biological activity of Lys was measured by a turbidimetric assay as described by Gorin et al. (1971), adopted for a plate reader, as described previously (Stanković et al., 2013). Since no biological assay is available for BSA, the absence of protein aggregation or denaturation was assessed semi-quantitatively, by the determination of additional peaks in the chromatograms assessed using the RP-HPLC method. Therefore, the HPLC method was used just as an indication of the protein intactness during the HME and release.

2.8. *Protein–polymer interaction*

As will be described in Section 3, 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 protein loaded implants were subjected to various assays (Table 4). 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) (Fenton and Fahey, 1986; Ghalanbor et al., 2012). 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 (Bilati et al., 2005).

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 and discussion

3.1. Polymer synthesis and characterization

The ε-caprolactone monomer conversion in the multiblock copolymer was 100%, while the D,L-lactide monomer conversion was 97%, indicating that a small percentage of D,L-lactide monomer may still be present in the multiblock copolymer. 1,4-Butanediol was used in low amounts as initiator molecule. H NMR spectra did not show any peaks representing residual unreacted 1,4-butanediol, which indicates quantitative (complete) conversion of 1,4-butanediol. The results of the characterization of *x*[PDLLA–PEG_{1000}]–*y*[PCL] multiblock copolymers by ^{1}H NMR, SEC and DSC are summarized in Table 3. SEC indicated that the apparent molecular weights of the various multiblock copolymers ranged from 15.4 to 21.3 kg/mol and 31–40 kg/mol, respectively. The polydispersity index, defined as the ratio M_w/M_n, ranged from 1.76–2.20.

From the ^{1}H NMR spectra it was calculated that polymerization of ε-caprolactone resulted in the formation of [PCL] with a M_n of 3500 g/mol, which was reasonably close to the theoretical value of 4000 g/mol, as calculated from in weight values and monomer conversion. The M_n of [PDLLA–PEG_{1000}] was 2000 g/mol, which was equal to the theoretical value of 2000 g/mol, as determined from in weights and monomer conversion.

Furthermore, ^{1}H NMR was used to verify the overall D,L-lactate/polyethylene glycol (LA/PEG) and ε-caprolactate/polyethylene glycol (CL/PEG) molar ratios of the multiblock copolymer. The overall LA/PEG molar ratio of the *x*[PDLLA–PEG_{1000}]–*y*[PCL] multiblock copolymers ranged from 14.5 to 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*[PDLLA–PEG_{1000}]–*y*[PCL] multiblock copolymers varied with varying PCL block content. CL/PEG molar ratios ranged from 16.8 to 135, as determined by ^{1}H NMR and were found close to the theoretical CL/PEG molar ratio as calculated from in weights (15.1–148) (Table 1). The NMR spectra of the prepolymers, and the multiblock copolymer 30[PDLLA–PEG_{1000}]–70[PCL] after extrusion and during degradation can be found in the supportive data section (Figs. S1 and S2). 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 (Bogdanov et al., 1998). The melting enthalpy (ΔH) of the multiblock copolymers was in the range of 61–74 J/g. The thermograms of the prepolymers [PCL] and [PDLLA–PEG_{1000}] and multiblock copolymer 30[PDLLA–PEG_{1000}]–70[PCL] can be found in the supportive data section (Figs. S3 and S4).

Supplementry material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.ijpharm.2015.01.007.

1,4-Dioxane was well removed from all *x*[PDLLA–PEG_{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_{1500}]–70[PCL] has been previously described (Stanković et al., 2013).

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*[PDLLA–PEG_{1000}]–*y*[PCL] polymers and 10.05 ± 0.01% (Lys) in 30[PCL–PEG_{1500}]–70[PCL] polymer. Both proteins showed absence of degradation or aggregation 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 and during the first 4 days of the release (data not shown). However, at the later data points the concentration of lysozyme from the medium was too low to be accurately tested by the biological assay.

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 [PDLLA–PEG_{1000}]/[PCL] block ratio and thus increased water-swellable 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%,

Table 3
^{1}H NMR, SEC and DSC results of *x*[PDLLA–PEG_{1000}]–*y*[PCL] multiblock copolymers.

Multiblock copolymers composition	LA/PEG molar ratio		CL/PEG molar ratio		SEC (kg/mol)			T_g [a] (°C)	T_m [a] (°C)	ΔH [a,b] (J/g)
	In weights	^{1}H NMR	In weights	^{1}H NMR	M_n	M_w	M_w/M_n			
10[PDLLA–PEG_{1000}]–90[PCL]	14.2	16.2	148.2	134.9	15.4	30.9	2.01	−54 ± 0.82	50 ± 1.05	72
20[PDLLA–PEG_{1000}]–80[PCL]	14.2	17.4	64.4	68.0	21.3	37.5	1.76	−49 ± 0.445	50 ± 0.59	74
30[PDLLA–PEG_{1000}]–70[PCL]	13.9	15.8	38.1	38.5	18.0	39.6	2.20	−40 ± 3.12	50 ± 0.40	74
50[PDLLA–PEG_{1000}]–50[PCL]	14.2	14.5	15.1	16.8	21.3	40.0	1.88	−39 ± 3.81	50 ± 0.02	61

[a] Determined after HME ($n = 2$).
[b] Total of the PCL fraction of the multiblock copolymers, calculated by ΔH (J/g PCL) = (ΔHm_{PCL}/wt% PCL), where ΔHm_{PCL} is the melting enthalpy of the PCL block per gram of multiblock copolymer, $wt\%_{PCL}$ is calculated by ^{1}H NMR.

59 ± 1.7%, 63 ± 0.7%, 68 ± 4.9% for 10[PDLLA–PEG_{1000}]–90[PCL], 20[PDLLA–PEG_{1000}]–80[PCL], 30[PDLLA–PEG_{1000}]–70[PCL], and 50[PDLLA–PEG_{1000}]–50[PCL], respectively (Fig. 1a). Water content of 30[PCL–PEG_{1500}]–70[PCL] after one day of incubation was 56 ± 1.9% and also remained constant during the entire study.

3.3.2. Mass loss

During incubation the mass loss of *x*[PDLLA–PEG_{1000}]–*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[PDLLA–PEG_{1000}]–90[PCL] was only 5.7 ± 0.1% after 180 days of incubation (Fig. 1b). With increased [PDLLA–PEG_{1000}]/[PCL] block ratio, and thus with an increased percentage of the water-swellable block, the percentage of mass loss increased amounting to 14.7 ± 0.7% for 20[PDLLA–PEG_{1000}]–80[PCL], 28.9 ± 0.9% for 30[PDLLA–PEG_{1000}]–70[PCL] and 50.6 ± 0.9% for 50[PDLLA–PEG_{1000}]–50[PCL] after 180 days of incubation. The reference copolymer, 30[PCL–PEG_{1500}]–70[PCL] having PCL in the amorphous block, showed only 8.4 ± 0.8% of mass loss during 140 days. The degradation of aliphatic polyesters is known to start upon water penetration into the amorphous regions of the polymer bulk, often accompanied by swelling, which induces hydrolysis of the ester bonds (Hu et al., 2004). 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 (Alexandra et al., 1997; Engineer et al., 2010; Göpferich, 1996).

For the *x*[PDLLA–PEG_{1000}]–*y*[PCL] multiblock copolymers with higher [PDLLA–PEG_{1000}]/[PCL] block ratio mass loss occurred immediately after 1 day of incubation, while for the copolymers with lower [PDLLA–PEG_{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 [PDLLA–PEG_{1000}]/[PCL] block ratio). Mass loss of the multiblock copolymer with [PCL–PEG_{1500}] (8.4% in 140 days) was slow as compared to copolymer with [PDLLA–PEG_{1000}] 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_{1500}] as compared to [PDLLA–PEG_{1000}] (Pitt et al., 1981b).

3.3.3. Decrease of molecular weight

It was found that during the first 14 days of incubation the M_n decreased faster when the [PDLLA–PEG_{1000}]/[PCL] block ratio of the multiblock copolymers was increased (Fig. 1c). The M_n of 10[PDLLA–PEG_{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 M_n of 20[PDLLA–PEG_{1000}]–80[PCL] already decreased to 60% of its original value, while for 30[PDLLA–PEG_{1000}]–70[PCL] and 50[PDLLA–PEG_{1000}]–50[PCL] the M_n decreased almost 70%. After the rapid decrease of the M_n of these copolymers during the first 14 days, the rate of M_n decrease declined and was similar for *x*[PDLLA–PEG_{1000}]–*y*[PCL] copolymers with an *x*/*y* ratio of 20/80, 30/70 and 50/50, resulting in 70–75% decrease in M_n after 180 days.

Contrary to multiblock copolymers containing PDLLA in the water-swellable block, 30[PCL–PEG_{1500}]–70[PCL] having PCL in the water-swellable block showed only 15% decrease in M_n during 140 days of incubation (Stanković et al., 2014).

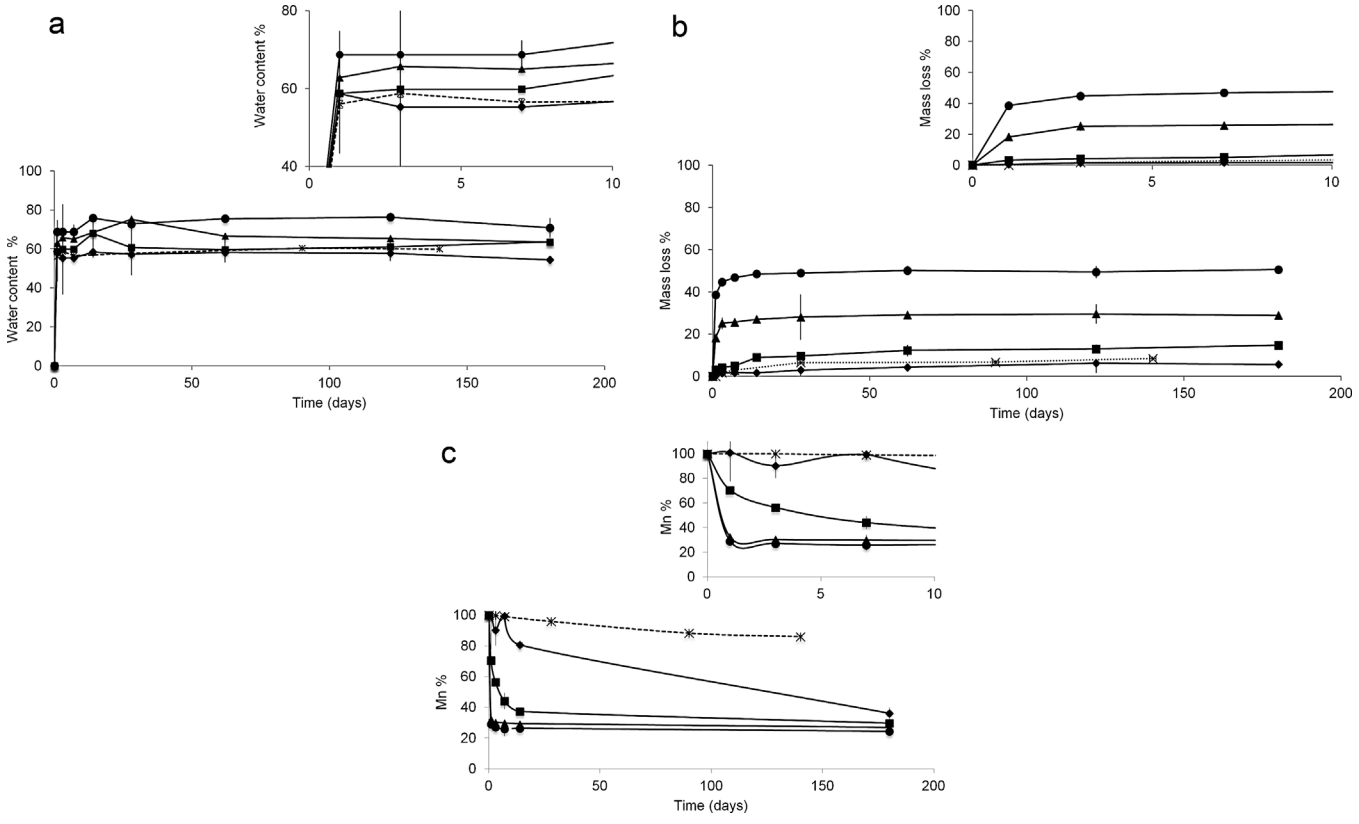

Fig. 1. Percentage of water content (a)*, mass loss (b) and (c) M_n loss for multiblock copolymers during degradation: 10[PDLLA–PEG_{1000}]–90[PCL] (◆), 20[PDLLA–PEG_{1000}]–80[PCL] (■), 30[PDLLA–PEG_{1000}]–70[PCL] (▲), 50[PDLLA–PEG_{1000}]–50[PCL] (●) and 30[PCL–PEG_{1500}]–70[PCL] (dashed line, ×). The error bars were included for all measurements; however, they were for some samples low and thus not visible. * Please note different *y* axis scale.

It is know from the literature that the poly(lactic acid) and poly (ε-caprolactone) polymers undergo bulk hydrolysis, where, depending on the degree of swelling of the material, auto-catalysis may occur due to accumulation of the acidic degradation products in the polymer matrix. Our results demonstrate that the molecular weight of the copolymers decreased immediately upon contact with water, which indicates bulk degradation of these copolymers. However, for polymers with higher amounts of the amorphous regions, the mass loss and thus the surface erosion contribute to degradation. This could be also observed on SEM figures (Fig. 4). The swellability and thus the degree of autocatalysis depended on the PEG content. The decrease in M_n was attributed to polymer chain hydrolysis, which occurred mainly in the water-swellable [PDLLA–PEG] block of the copolymer. Polyesters degrade by hydrolysis of the ester bonds (Erlandsson et al., 2000; Grizzi et al., 1995; Jonnalagadda and Robinson, 2004). Additionally, the ester bonds in PDDLA are more susceptible to hydrolysis than the ester bonds in PCL, which makes this polymer to degrade faster than PCL. The preferential degradation of the [PDLLA–PEG] blocks is supported by ^{1}H NMR data, which showed that the content of lactic acid and PEG (and thus the content of the [PDLLA–PEG] block) decreased and that the relative amount of PCL increased during degradation. Also, in line with these findings, for all *x* [PDLLA–PEG_{1000}]–*y*[PCL] copolymers the percentage of mass loss did not exceed the mass percentage of the amorphous block in the copolymer. Therefore, it can be assumed that the degradation products of these copolymers in the aqueous medium will consist mainly of PEG–PDDLA oligomers, water-soluble PDLLA oligomers and lactic acid with minor amount of water-soluble PCL oligomers and caprolactic acid. Further, ^{1}H NMR also showed that the urethane bond content of the copolymers decreased in time. Thus, besides the above listed components, the aqueous medium will also contain at least water-soluble butane–urethane bond-PDLLA (oligomers) and possibly, after relatively slow hydrolysis of the urethane bond, butanediamine.

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 Fig. 2, during the initial stages of degradation, the melting enthalpy of the PCL blocks of the various [PDLLA–PEG_{1000}]–[PCL] polymers increased from 60 to 70 J/g to approximately 80–90 J/g. The independence of PCL melting enthalpy on PCL content is due to the phase-separated nature of the copolymer, where the crystalline PCL domains are phase-separated from the amorphous PEG/PDLLA/amorphous PCL domains. As shown from the water uptake and mass loss data, the amorphous regions of [PDLLA–PEG_{1000}] and amorphous regions of [PCL] degraded faster than the crystalline regions of PCL, resulting in overall increased crystallinity of the PCL during degradation of the polymers. All the DSC measurements were performed in dry state. In aqueous medium, PEG will lose its crystallinity and will be in the dissolved state. For further information about the thermograms of PCL and PDDL–PEG prepolymers, as well as 30[PDLA–PEG_{1000}]–70[PCL] multiblock copolymer after synthesis, reference is made to the Supplementary data section (Figs. S3 and S4).

3.3.5. Polymer composition

^{1}H NMR showed that the relative ε-caprolactone content of all four *x*[PDLLA–PEG_{1000}]–*y*[PCL] multiblock copolymers increased during incubation, while the PEG and PDLLA contents decreased, indicating that degradation mainly occurred in the amorphous blocks. The ^{1}H NMR data for a representative polymer, 30[PDLLA–PEG_{1000}]–70[PCL], are shown in Fig. 3. Within 10 days, the lactic acid content of this polymer had decreased from its original 15 wt% to 0 wt%. In contrast to the multiblock copolymers containing PDLLA in the water-swellable block, the composition of 30[PCL–PEG_{1500}]–70[PCL], having PCL in the water-swellable block, did not substantially change (data not shown).

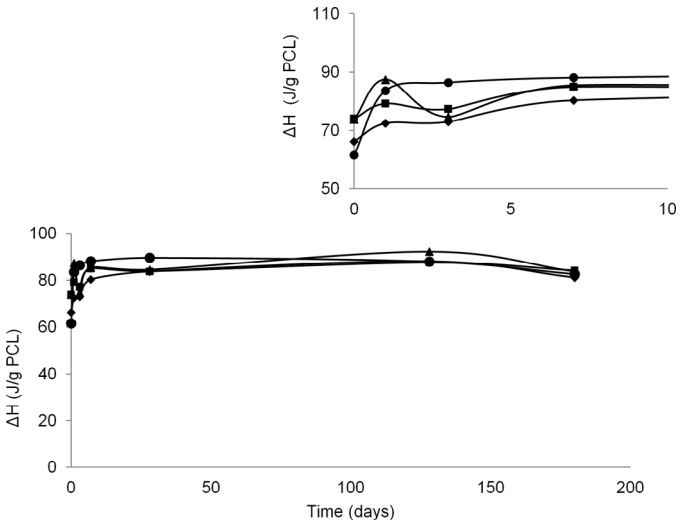

Fig. 2. The melting enthalpy of the crystalline [PCL] of the multiblock copolymers during degradation, corrected for the total PCL content ($n = 2$), 10[PDLLA–PEG_{1000}]–90[PCL] (◆), 20[PDLLA–PEG_{1000}]–80[PCL] (■), 30[PDLLA–PEG1000]–70[PCL] (▲), 50[PDLLA–PEG1000]–50[PCL] (●). The error bars were included for all measurements; however, they were for some samples low and thus not visible.

3.3.6. Surface morphology

The surface of the [PDLLA–PEG_{1000}] based polymeric implants was examined after 180 days of degradation by SEM. As can be seen in Fig. 4, 10[PDLLA–PEG_{1000}]–90[PCL] implants appeared to be intact even after 180 days of incubation. The surface of the 20 [PDLLA–PEG_{1000}]–80[PCL] implants appeared more degraded as it showed the presence of ruptures. The 30[PDLLA–PEG_{1000}]–70[PCL] implants exhibited surface roughness, indicating that they were degraded to a more advanced stage than the two implants with lower [PDLLA–PEG_{1000}]/[PCL] block ratio. Finally, 50[PDLLA–PEG_{1000}]–50[PCL] implants were heavily shrunken after 180 days, indicating substantial degradation. Even though bulk degradation is considered a main mechanism involved in the degradation of these copolymers, the affected surface morphology of the 50 [PDLLA–PEG_{1000}]–50[PCL] implants implies a potential contribution of surface erosion to the degradation mechanism. The surface of the 30[PCL–PEG_{1500}]–70[PCL] implant showed no visible signs of degradation.

3.3.7. In-vitro protein release

The burst release of BSA and Lys from *x*[PDLLA–PEG_{1000}]–*y*[PCL] copolymers largely depended on the [PDLLA–PEG_{1000}]/[PCL] block ratio (and thus on the PEG content) of the polymer (Fig. 5). With increasing [PDLLA–PEG_{1000}]/[PCL] block ratio, the burst release increased. For the implants with the highest water-swellable block content, i.e., 50[PDLLA–PEG_{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 *x*[PDLLA–PEG_{1000}]–*y*[PCL] based formulations. In contrast, 30[PCL–PEG_{1500}]–70[PCL], containing PCL instead of PDLLA in the water-swellable 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). It has previously been described that burst release of protein is most likely due to

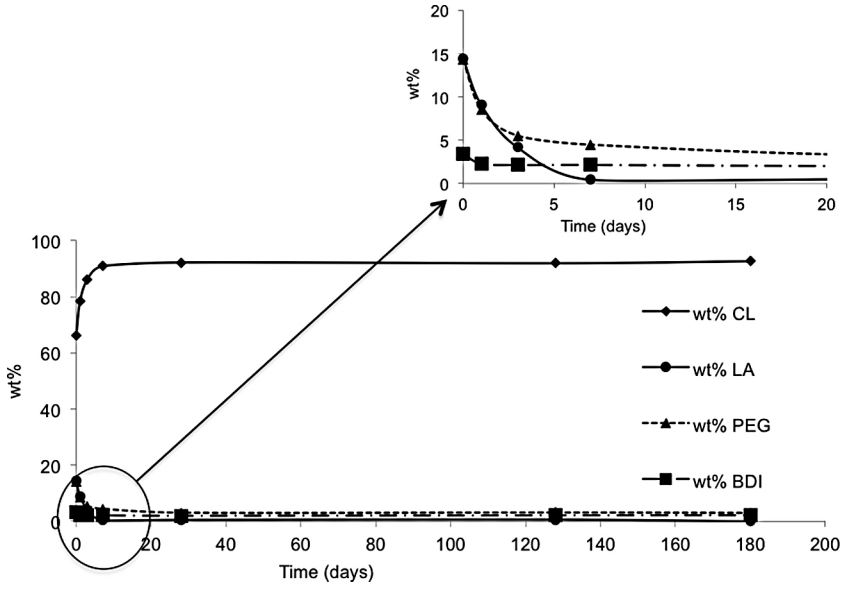

Fig. 3. Relative change of wt% of various components of 30[PDLLA–PEG_{1000}]–70[PCL] implant during degradation, compared to day 0.

dissolution of particles from the polymer surface followed by dissolution and liberation of the neighboring (percolating) particles (Stanković et al., 2013). In this study, we observed that the burst release increased with increasing [PDLLA–PEG_{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 [PDLLA–PEG_{1000}]/[PCL] block ratio of 30/70 and 50/50 exhibited a certain mass loss already during the burst phase, indicating that the increased burst release with increased [PDLLA–PEG_{1000}]/[PCL] block ratio may also be ascribed to liberation of the proteins together with parts of the [PDLLA–PEG_{1000}] blocks of the polymer.

As shown earlier (Stanković et al., 2014) for low swellable polymers, the molecular weight of the protein is an important factor that affects the release rate. Proteins of a higher molecular weight will not be released unless polymer degradation occurs. Hence, with a high content of water-swellable [PDLLA–PEG_{1000}] block and thus enhanced polymer swelling, the release is mainly driven by dissolution/degradation of the [PDLLA–PEG_{1000}] block leading to release of both small and large proteins. In the present study, we showed that, except for Lys being completely released from 50[PDLLA–PEG_{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*[PDLLA–PEG_{1000}]–*y*[PCL] copolymers. Next to the particle size and protein molecular weight, the hydrophilicity of the protein may also affect to the way it is incorporated into the polymer matrix. Because the proteins used in this study are rather hydrophilic, it is most likely that they are preferentially incorporated into the hydrophilic regions of the copolymer (PDDLA–PEG blocks). However, due to viscous flow of the polymer and limited diffusional mobility of the protein during the HME process, the proteins may also have been partially dispersed in the hydrophobic regions of the copolymer (PCL blocks). This could explain that even after dissolution of the semi-crystalline PLA–PEG block, not all protein was released, which will be further discussed below.

As mentioned earlier, immediately after extrusion quantitative recovery of the proteins from both *x*[PDLLA–PEG_{1000}]–*y*[PCL] and 30[PCL–PEG_{1500}]–70[PCL] implants was possible after extraction of the polymer using 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*[PDLLA–PEG_{1000}]–*y*[PCL] implants could not be achieved. Attempts to dissolve polymer of the protein containing *x*[PDLLA–PEG_{1000}]–*y*[PCL] implants in other organic solvents or solvent systems (acetone, acetonitrile, DMSO/0.05 N NaOH + 0.5% SDS, dichloromethane), which are described in literature as suitable solvents for similar polymeric systems (Sah, 1997; Stanković et al., 2014; Zhu and Schwendeman, 2000) were unsuccessful as well. It was hypothesized that irreversible interactions of the proteins with degrading polymer and 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. To confirm this hypothesis, the polymer only implants (without incorporated proteins) prepared from 30[PDLLA–PEG_{1000}]–70[PCL] were collected after 28 days of degradation, dried for 24 h in a vacuum dessicator and immersed in ethyl acetate and dichloromethane. Contrary to the protein loaded implants, the polymer only implants were fully soluble in both solvents, which confirmed that the poor solubility can indeed be related to the presence of protein in the degrading polymer matrix.

In contrast to the *x*[PDLLA–PEG_{1000}]–*y*[PCL] implants, the release of Lys from 30[PCL–PEG_{1500}]–70[PCL] implant was continuous during the entire duration of the release study. Furthermore, 30[PCL–PEG_{1500}]–70[PCL] based Lys containing implants could be dissolved almost completely in ethyl acetate after 35 days of incubation. As 30[PCL–PEG_{1500}]–70[PCL] only moderately degrades during the period of incubation (Stanković et al., 2014), the incomplete release from the [PDLLA–PEG_{1000}]–[PCL] copolymers can be related to the degradation of these polymers.

3.4. Interaction of protein and polymer

Incomplete protein release from biodegradable polymer matrices has been observed before, but little is known about the exact mechanism. Compromised protein release has been ascribed to protein adsorption to the polymer and protein aggregation (Jiang et al., 2002; Nam et al., 2000; Zhu and Schwendeman, 2000). Other authors reported that incomplete peptide or protein release can be due to the chemical modification of proteins during incubation, e.g., by acylation (Crotts and Park, 1998; Ghalanbor

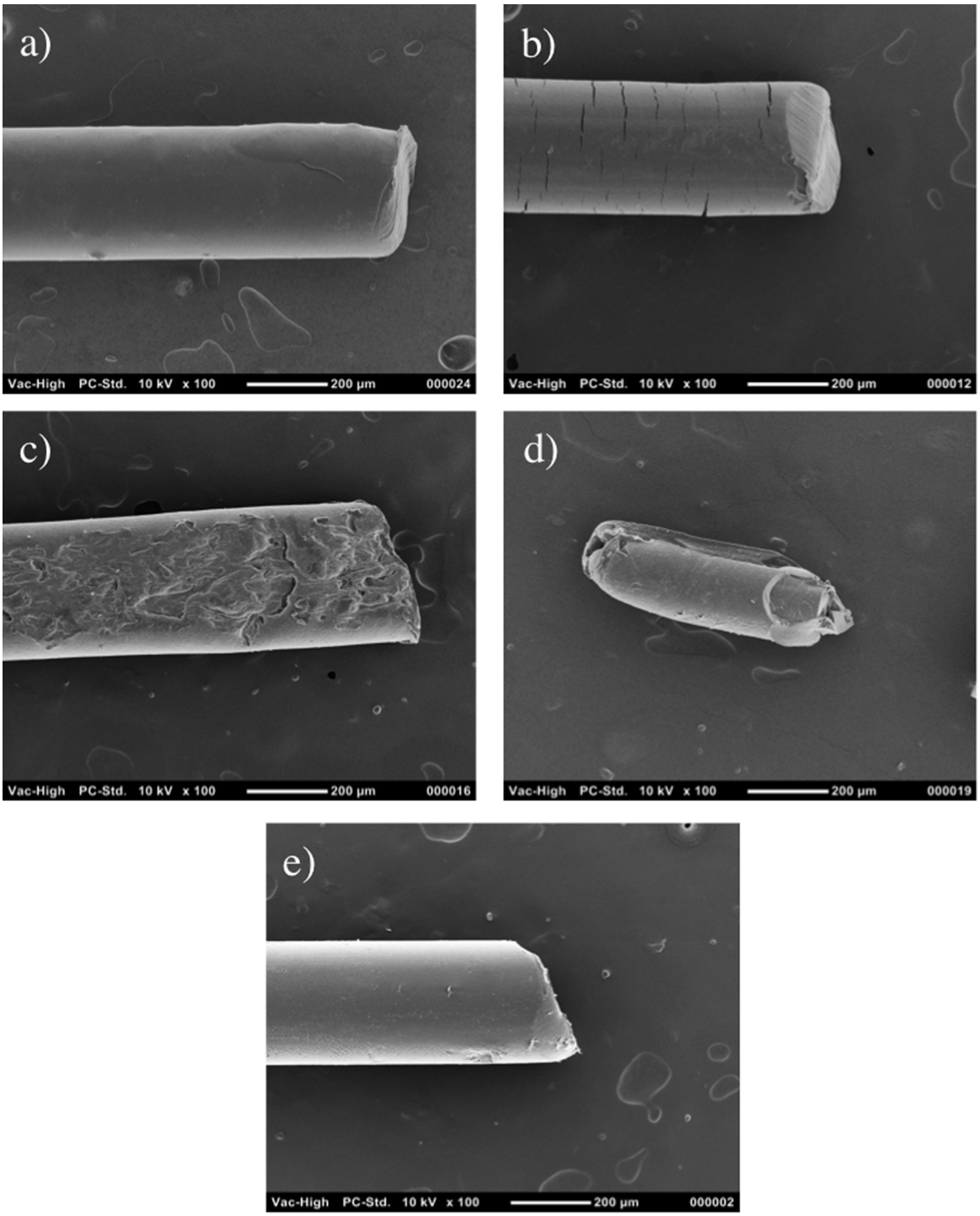

Fig. 4. Surface of the polymer-only implants after 180 days of incubation in PBS. (a) 10[PDLLA–PEG_{1000}]–90[PCL]; (b) 20[PDLLA–PEG_{1000}]–80[PCL]; (c) 30[PDLLA–PEG_{1000}]–70[PCL], (d) 50[PDLLA–PEG_{1000}]–50[PCL], (e) 30[PCL–PEG_{1500}]–70[PCL] (140 days of incubation).

et al., 2012; Houchin et al., 2006; Na et al., 2003; Park et al., 1995). In order to assess the nature of the interaction between protein and polymer, the implants that showed incomplete release were collected after 17 days from the release buffer and were exposed to aqueous solutions of urea, SDS, DTT, hydroxylamine HCL and NaOH for one day, after which the amount of the released protein was determined (Table 4).

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. It was shown that only incubation in 1 M NaOH resulted in an additional 50% release of the both incorporated BSA and Lys most likely due to the ability of NaOH to hydrolyze the ester bonds and completely degrade the polymer. These findings suggest that the reason for incomplete release was not disulfide bridge formation, nor aggregation/denaturation but irreversible interaction or covalent bonding between the protein and [PDLLA–PEG_{1000}] related degradation products. However, it has also been observed that after a few days of incubation, the crystallinity of PCL was increased indicating molecular rearrangements of PCL of the semi-

Table 4
Cumulative amount of protein released after 17 days of incubation of protein-loaded 30[PDLLA–PEG_{1000}]–70[PCL] implants in PBS. Further release of protein upon addition of different reagents and incubation for 24 h.

	% Released		% Released		% Released		% Released		% Released	
	Lys	BSA	Lys	BSA	Lys	BSA	Lys	BSA	Lys	BSA
t = 17 days	43.00	43.10	43.29	45.96	40.73	45.81	45.26	48.11	40.66	45.53
Addition of:	NaOH		Urea6M		DTT		SDS		Hydroxylamine HCl	
t = 24 h	93.60	93.63	46.57	47.40	39.57	45.94	45.51	50.21	41.21	45.94

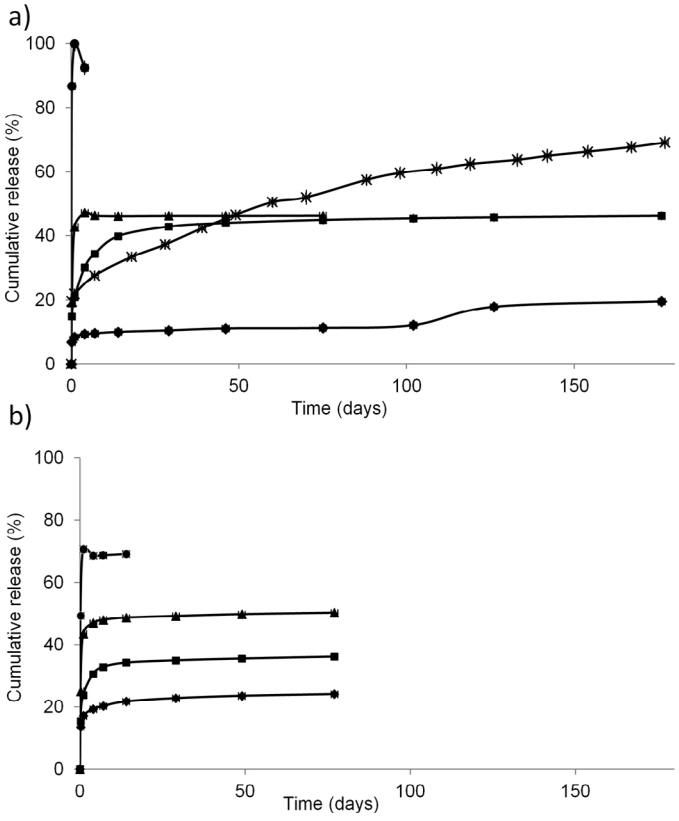

Fig. 5. *In-vitro* release of Lys (a) and BSA (b) from multiblock copolymers (n = 3): 10[PDLLA–PEG_{1000}]–90[PCL] (◆), 20[PDLLA–PEG_{1000}]–80[PCL] (■), 30[PDLLA–PEG_{1000}]–70[PCL] (▲), 50[PDLLA–PEG_{1000}]–50[PCL] (●) and 30[PCL–PEG_{1500}]–70[PCL] (×). Please note that the 50[PDLLA–PEG_{1000}]–50[PCL] line overlaps with the y axis. The error bars were included for all measurements; however, they were for some samples low and thus not visible.

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 bound to the polymer degradation products in the implant, which has also been shown before (Quaglia et al., 2005). However, if the protein would remain physically entrapped, it should have been recovered upon the protein extraction using ethyl acetate.

4. Conclusions

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_{1500}] by rapidly degrading [PDLLA–PEG_{1000}] block. We have shown that copolymers composed of x[PDLLA–PEG_{1000}]–y[PCL] exhibit continuous degradation rate in the initial phase, which can be controlled by varying the [PDLLA–PEG_{1000}]/[PCL] block ratio. According to our expectations, faster degradation resulted in accelerated protein release. However, incomplete protein release was observed, which limited the application of these polymers for sustained release formulations. We concluded that either irreversible interaction between the protein and degradation products originating from the [PDLLA–PEG_{1000}] block or the physical entrapment of the protein into the semi-crystalline hydrophobic PCL matrix compromised protein release. Even though proteins used in this study will most likely be incorporated into the hydrophilic regions of the polymers, during the preparation of implants by HME process, due to the shear forces and rearrangement of the molecular chains of polymers, proteins might be partially incorporated into the more hydrophobic polymer regions as well. During release, after the amorphous regions are dissolved and the crystallinity of the polymer is slightly increased, the protein might remain entrapped in the hydrophobic polymer regions.

Contrary, protein release from the slowly degrading x[PCL–PEG_{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 toward the successful development of protein loaded polymer depot formulations should be directed toward elucidation of the exact mechanism of incomplete protein release and toward the fundamental understanding of the molecular interaction between protein and polymer degradation products.

Acknowledgement

This research has been performed within the framework of the Northern Drug Targeting and Delivery Cluster (EFRO Grant).

References

Alexandra, R.-W., Besseghir, K., Gurny, R., 1997. Analysis of the influence of polymer characteristics and core loading on the in vivo release of a somatostatin analogue. Eur. J. Pharm. Sci. 5, 303–313.
Bakri, S.J., Snyder, M.R., Reid, J.M., Pulido, J.S., Ezzat, M.K., Singh, R.J., 2007. Pharmacokinetics of intravitreal ranibizumab (Lucentis). Ophthalmology 114, 2179–2182. doi:http://dx.doi.org/10.1016/j.ophtha.2007.09.012.
Bilati, U., Allémann, E., Doelker, E., 2005. Strategic approaches for overcoming peptide and protein instability within biodegradable nano- and microparticles. Eur. J. Pharm. Biopharm. 59, 375–388.
Bogdanov, B., Vidts, A., Van Den Bulcke, A., Verbeeck, R., Schacht, E., 1998. Synthesis and thermal properties of poly(ethylene glycol)-poly(ε-caprolactone) copolymers. Polymer 39, 1631–1636.
Chen, W., Luo, W., Wang, S., Bei, J., 2003. Synthesis and properties of poly(L-lactide)-poly(ethylene glycol) multiblock copolymers by coupling triblock copolymers. Polym. Adv. Technol. 14, 245–253.
Chen, H., Yuan, L., Song, W., Wu, Z., Li, D., 2008. Biocompatible polymer materials: role of protein–surface interactions. Prog. Polym. Sci. 33, 1059–1087. doi:http://dx.doi.org/10.1016/j.progpolymsci.2008.07.006.
Crotts, G., Park, T.G., 1998. Protein delivery from poly(lactic-co-glycolic acid) biodegradable microspheres: release kinetics and stability issues. J. Microencapsul. 15, 699–713.
Engineer, C., Parikh, J., Raval, A., 2010. Hydrolytic degradation behavior of 50/50 poly lactide-co-glycolide from drug eluting stents. Trends Biomater. Artif. Organs 24, 131–138.
Erlandsson, B., Renstad, R., Albertsson, A., Karlsson, S., Eldsa, C., 2000. The biodegradation of amorphous and crystalline regions in film-blown poly(e-caprolactone). Polymer 41, 1297–1304.

Fenton, S.S., Fahey, R.C., 1986. Analysis of biological thiols: determination of thiol components of disulfides and thioesters. Anal. Biochem. 154, 34–42.
Frank, A., Rath, S.K., Venkatraman, S.S., 2005. Controlled release from bioerodible polymers: effect of drug type and polymer composition. J. Control. Release 102, 333–344.
Freiberg, S., Zhu, X.X., 2004. Polymer microspheres for controlled drug release. Int. J. Pharm. 282, 1–18.
Göpferich, A., 1996. Mechanisms of polymer degradation and erosion. Biomaterials 17, 103–114.
Ghalanbor, Z., Körber, M., Bodmeier, R., 2010. Improved lysozyme stability and release properties of poly(lactide-co-glycolide) implants prepared by hot-melt extrusion. Pharm. Res. 27, 371–379.
Ghalanbor, Z., Körber, M., Bodmeier, R., 2012. Protein release from poly(lactide-co-glycolide) implants prepared by hot-melt extrusion: thioester formation as a reason for incomplete release. Int. J. Pharm. 438, 302–306.
Gorin, G., Wang, S., Papapavlou, L., 1971. Assay of lysozyme by its lytic action on M. lysodeikticus cells. Anal. Biochem. 39, 113–127.
Grizzi, I., Garreau, H., Li, S., Vert, M., 1995. Hydrolytic degradation of devices based on poly(DL-lactic acid) size-dependence. Biomaterials 16, 305–311. doi:http://dx.doi.org/10.1016/0142-9612(95)93258-F.
Houchin, M.L., Heppert, K., Topp, E.M., 2006. Deamidation, acylation and proteolysis of a model peptide in PLGA films. J. Control. Release 112, 111–119.
Hu, Y., Zhang, L., Cao, Y., Ge, H., Jiang, X., Yang, C., 2004. Degradation behavior of poly (epsilon-caprolactone)-b-poly(ethylene glycol)-b-poly(epsilon-caprolactone) micelles in aqueous solution. Biomacromolecules 5, 1756–1762.
Huang, M.-H., Li, S., Hutmacher, D.W., Coudane, J., Vert, M., 2006. Degradation characteristics of poly(epsilon-caprolactone)-based copolymers and blends. J. Appl. Polym. Sci. 102, 1681–1687.
Jain, R.A., 2000. The manufacturing techniques of various drug loaded biodegradable poly(lactide-co-glycolide) (PLGA) devices. Biomaterials 21, 2475–2490.
Jiang, G., Woo, B.H., Kang, F., Singh, J., DeLuca, P.P., 2002. Assessment of protein release kinetics, stability and protein polymer interaction of lysozyme encapsulated poly(D,L-lactide-co-glycolide) microspheres. J. Control. Release 79, 137–145.
Jonnalagadda, S., Robinson, D.H., 2004. Effect of thickness and PEG addition on the hydrolytic degradation of PLLA. J. Biomater. Sci. Polym. Ed. 15, 1317–1326. doi:http://dx.doi.org/10.1163/1568562041959982.
Li, S., Garreau, H., Vert, M., Petrova, T., Manolova, N., Rashkov, I., 1997. Hydrolytic degradation of poly(oxyethylene)-poly-(ε-caprolactone) multiblock copolymers. J. Appl. Polym. Sci. 68, 989–998.
Liao, Y.H., Brown, M.B., Martin, G.P., 2001. Turbidimetric and HPLC assays for the determination of formulated lysozyme activity. J. Pharm. Pharmacol. 53, 549–554.
Lucke, A., Teßmar, J., Schnell, E., Schmeer, G., Göpferich, A., 2000. Biodegradable poly (D,L-lactic acid)-poly(ethylene glycol)-monomethyl ether diblock copolymers: structures and surface properties relevant to their use as biomaterials. Biomaterials 21, 2361–2370.
Na, D.H., Youn, Y.S., Lee, S.D., Son, M.-W., Kim, W.-B., DeLuca, P.P., Lee, K.C., 2003. Monitoring of peptide acylation inside degrading PLGA microspheres by capillary electrophoresis and MALDI-TOF mass spectrometry. J. Control. Release 92, 291–299. doi:http://dx.doi.org/10.1016/S0168-3659(03)00366-3.
Nam, Y.S., Song, S.H., Choi, J.Y., Park, T.G., 2000. Lysozyme microencapsualtion within biodegradable PLGA microspheres: urea effect on protein release and stability. Biotechnol. Bioeng. 70, 270–277.
Park, T.G., Lu, W., Crotts, G., 1995. Importance of in vitro experimental conditions on protein release kinetics, stability and polymer degradation in protein encapsulated poly(D,L-lactic acid-co-glycolic acid) microspheres. J. Control. Release 33, 211–222.
Park, J.H., Ye, M., Park, K., 2005. Biodegradable polymers for microencapsulation of drugs. Molecules 10, 146–161.
Pitt, C.G., Chasalow, F.I., Hibionada, Y.M., Klimas, D.M., 1981a. Aliphatic polyesters I. The degradation of poly(ε-caprolactone) in vivo. J. Appl. Polym. Sci. 26, 3779–3787.
Pitt, C.G., Gratzl, M.M., Kimmel, G.L., Surles, J., Schindler, A., 1981b. Aliphatic polyesters II. The degradation of poly(DL-lactide) poly(epsilon-caprolactone), and their copolymers in vivo. Biomaterials 2, 215–220.
Quaglia, F., Ostacolo, L., Nese, G., De Rosa, G., La Rotonda, M.I., Palumbo, R., Maglio, G., 2005. Microspheres made of poly(epsilon-caprolactone)-based amphiphilic copolymers: potential in sustained delivery of proteins. Macromol. Biosci. 5, 945–954. doi:http://dx.doi.org/10.1002/mabi.200500108.
Rashkov, I., Manolova, N., Li, S.M., Espartero, J.L., Vert, M., 1996. Synthesis, characterization, and hydrolytic degradation of PLA/PEO/PLA triblock copolymers with short poly(L-lactic acid) chains. Macromolecules 29, 50–56.
Roach, P., Farrar, D., Perry, C.C., 2005. Interpretation of protein adsorption: surface-induced conformational changes. J. Am. Chem. Soc. 127, 8168–8173. doi:http://dx.doi.org/10.1021/ja042898o.
Sah, H., 1997. A new strategy to determine the actual protein content of poly(lactide-co-glycolide) microspheres. J. Pharm. Sci. 86, 1315–1318.
Seyednejad, H., Ghassemi, A.H., Nostrum, C.F., Van Vermonden, T., Hennink, W.E., 2011. Functional aliphatic polyesters for biomedical and pharmaceutical applications. J. Control. Release 152, 168–176.
Stanković, M., De Waard, H., Steendam, R., Hiemstra, C., Zuidema, J., Frijlink, H.W., Hinrichs, W.L.J., 2013. Low temperature extruded implants based on novel hydrophilic multiblock copolymer for long-term protein delivery. Eur. J. Pharm. Sci. 49, 578–587.
Stanković, M., Tomar, J., Hiemstra, C., Steendam, R., Frijlink, H.W., Hinrichs, W.L.J., 2014. Tailored protein release from biodegradable poly(ε-caprolactone-PEG)-b-poly(ε-caprolactone) multiblock-copolymer implants. Eur. J. Pharm. Biopharm. 87, 329–337.
Sun, H., Mei, L., Song, C., Cui, X., Wang, P., 2006. The in vivo degradation, absorption and excretion of PCL-based implant. Biomaterials 27, 1735–1740.
The International Organization for Standardization, 1999. Implants for surgery—copolymers and blends based on polylactide—in vitro degradation and testing. ISO/FDIS 15814, Geneva . https://www.iso.org/obp/ui/#iso:std:iso:15814:ed-1:v1:en (accessed 11.02.14).
Vert, M., Li, S., Garreau, H., 1991. More about the degradation of LA/GA-derived aqueous media matrices in aqueous media. J. Control. Release 16, 15–26.
Vert, M., 2005. Aliphatic polyesters: great degradable polymers that cannot do everything. Biomacromolecules 6, 538–546.
Vilar, G., Tulla-Puche, J., Albericio, F., 2012. Polymers and drug delivery systems. Curr. Drug Deliv. 9, 367–394.
Zhu, G., Schwendeman, S.P., 2000. Stabilization of proteins encapsulated in cylindrical poly(lactide-co-glycolide) implants: mechanism of stabilization by basic additives. Pharm. Res. 17, 351–357.